



Utilization of Emulsiflex Prepared *Escherichia coli* Strain EC4 Oxyrase for Improved Cultivation of Anaerobic Bacteria by using Hungate Technique and Determination of its Kinetic Parameters

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ABSTRACT

Present study was conducted for the utilization of Emulsiflex prepared oxyrase extracted from cytoplasmic of membrane fragments of *E. coli*. For this purpose, 88 *E. coli* isolates from 30 intact poultry intestines were cultured and identified preliminary on the basis of microscopy and biochemical testing. *E. coli* strain EC4 was screened as best oxyrase producer with activity of $0.41 \pm 0.008 \text{ U/mL/min}$ with 41% reduction in dissolved oxygen at pH 7.5, temperature 37°C , 25mM lactate as H^+ donor after 20 min. This strain was later on confirmed by 16S ribotyping procedure. Oxyrase was used for improved cultivation of anaerobic bacteria by employing Hungate Technique where its antioxidant potential was compared with common reducing agent Cysteine-HCl (Cys-HCl). 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 $\text{OD}_{600\text{nm}}$ of 0.80 ± 0.0017 reached after 4 days of incubation when oxyrase used as reducing agent, and maximum $\text{OD}_{600\text{nm}}$ 0.65 ± 0.0016 in presence of Cys-HCl. *Akkermansia muciniphila* culture reached maximum $\text{OD}_{600\text{nm}}$ 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 $\text{OD}_{600\text{nm}}$ 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 $\text{OD}_{600\text{nm}}$ of 2.0 ± 0.05 after 73 h of incubation. Whereas, in the presence of Cys-HCl *Bilophila wadsworthia* cells only achieved maximum $\text{OD}_{600\text{nm}}$ 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 $\text{OD}_{600\text{nm}}$ of 2.1 ± 0.06 after 25 h of incubation. With Cys-HCl, maximum cell density of *Roseburia intestinalis* with $2.0 \pm 0.06 \text{ OD}_{600\text{nm}}$ was observed after 73 h. Kinetic parameters derived from double reciprocal Lineweaver-Burk plot showed K_m values $8.49 \times 10^{-3} \text{ M}^{-1}$ and $4.75 \times 10^{-3} \text{ M}^{-1}$ of sonication and Emulsiflex prepared oxyrase membrane fragments, respectively for lactate as substrate.

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Authors' Contribution

MUA conceived, designed and performed the experimental work. MUA and IUH prepared the manuscript. IUH supervised the experimental work.

Key words

Oxyrase, *Escherichia coli* strain EC4, Hungate technique, Cytoplasmic membrane fragments and Emulsiflex.

INTRODUCTION

Certain enzyme systems in cytoplasmic membrane fragments of some bacteria can efficiently catalyze the enzymatic reduction of dissolved oxygen (DO) in medium to H_2O . Recent studies have drawn attention towards practical applications of these membrane fragments for reduction of DO to water by a terminal chain reaction in electron transport system of aerobic respiration (Palmer and Bonner, 2007). Various studies have reported several microorganisms for production of oxygen reducing cytoplasmic membrane fragments, such as *Escherichia coli*, *Gluconobacter oxydans*, *Pseudomonas aeruginosa*,

Mycobacterium phlei, *Azotobacter vinlandii* and *Salmonella typhimurium* (Ahmad et al., 2017; Adler, 1990).

E. coli is a gram-negative, rod-shaped, facultative anaerobic, non-spore forming bacterium which sometimes may form a capsule. Basic biochemical characteristics of this Enterobacterium are oxidase negative and catalase positive, as well as ability to ferment glucose and reduction of nitrates to nitrites. *E. coli* is a common inhabitant of poultry intestinal tracts at numbers up to $10^6/\text{g}$ of fecal material. Birds without an established microflora, younger birds, and lower intestinal tract reportedly possess higher densities of *E. coli* (Jin et al., 1997). Poultry waste is regarded as a rich source of bacterial flora with ability to express array of enzymes (Rehman and Imtiaz, 2018).

Ribotyping is one of the frequently used methods to study taxonomy and bacterial phylogeny. It utilizes 16S rRNA

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gene sequences as most common genetic marker for various reasons, such as: its gene size of 1500 bp is large enough for informatics analysis; non-redundancy of its function; its highly conservative existence as multigene family or operons in almost all bacteria (Janda and Abbott, 2007).

Avestin Emulsiflex-C3® is very efficient, with capacity of 3L/h, and easy to use for efficient *E. coli* cell lysis in one passage (with 90% efficiency) at 100 MPa (15,000 psi) in laboratory setups (Andrews and Asenjo, 1987). Concomitant decrease of redox potential from 200 to 300 mV by utilizing oxygen reducing membrane fragments to complex microbiological media has been reported. Without utilizing any other chemical reducing agent, small amounts of sterile *E. coli* cytoplasmic membranes are being added to bacteriological media for cultivation of anaerobic bacterial strains. These preparations comprising of *E. coli* membrane fragments can reduce DO in few minutes after they were added to create microanaerobic environments. By any means during incubation, diffusing oxygen in medium from the head space gets rapidly reduced to water and hence keeping it anaerobic (Adler and Spady, 1997).

DO can be detected in the medium by various methods like electrochemical (potentiometric, amperometric, or conductometric), chemical (Winkler) and optical (photoluminescence). But since the development of Clark Electrode/Probe based method, it has been widely used for measurement of DO because of its reliability and sensitivity (Quaranta *et al.*, 2012).

Obligate anaerobes are those microorganisms which are not able to utilize molecular oxygen for their growth. Hungate technique is one of the recommended methods for culturing of anaerobic bacteria in research laboratories. *Anaerobaculum hydrogeniformans* is a moderately thermophilic, anaerobic, NaCl-requiring, Gram-negative, non-spore forming, fermentative, rod-shaped bacterium. Strain OS1 has been originally isolated from oil production water reserves of Alaska, USA (Maune and Tanner, 2012). *Akkermansia muciniphila* is an oval-shaped, non-motile, non-spore-forming, gram-negative, and strictly anaerobic bacterium (Dao *et al.*, 2016). *Bilophila wadsworthia* is recognized as obligate anaerobic, nonspore forming, rod shaped gram-negative bacterium (Sawamura *et al.*, 1997). *Roseburia intestinalis* is gram-positive, anaerobic, slightly curved rod shaped bacterium and exhibit motility by multiple sub-terminal flagella (Duncan, 2002).

Present work was aimed at utilizing Emulsiflex for the first time to prepare oxygen reducing cytoplasmic membrane fragments from *Escherichia coli* strain EC4. Immense antioxidant potential of oxyrase enzyme system in these membrane fragments was evaluated. Combination of oxyrase as reducing agent and the Hungate technique for cultivation of anaerobic bacteria was used for this purpose.

MATERIALS AND METHODS

Isolation, preliminary identification and screening of oxyrase producing E. coli

Thirty intact poultry intestines were collected in a clean decontaminated polyethylene zipper bag, from 30 sites/broiler poultry butcher shops located in three different areas: Ichhra, Anarkali and Rehmanpura Lahore. Approximately three inches of distal colon at first point proximal to rectum, which contained feces, was resected with sterile scalpel and all fecal material was aseptically transferred to 10mL sterile phosphate-buffered saline (pH 7.4) in test tubes (Feder *et al.*, 2003). Fecal material in test tubes was mixed/homogenized by repeated pipetting.

Eosin methylene blue (EMB) agar medium plates were inoculated with 100µL diluted samples by spread plate method on and incubated at 37°C for 24 h. Quadrant streak plate method was used for pure culturing of *E. coli*. All different isolates from various samples were separately maintained on nutrient agar (pH 6.8) slants for further identification.

Loop smears of isolates were prepared from *E. coli* characteristic metallic-sheen green colonies on EMB agar master plates. Gram staining of smears was performed according to Cheesbrough (2006). Microscopic observations of stained smears were done at 100X objective using OLYMPUS® CH30 BINOCULAR Microscope. Various recommended biochemical tests were performed for preliminary identification of isolates as *E. coli* (Holt *et al.*, 1994; Cheesbrough, 2006). Twenty four hours old cultures of isolates were used for biochemical identification. Upstream, downstream process for screening of oxyrase for *E. coli* isolates was setup according to methodology described in our previous work (Ahmad *et al.*, 2017).

Quantitative determination of oxyrase units

Benchtop dissolved oxygen meter was used for quantification of oxyrase according to manufacturer's protocol. Reaction suspension with total volume of 5.0mL was constituted containing 500µL of membrane preparation + 4.5mL of phosphate buffer (pH 7.5) was incubated at 37°C for 3 min. 10mM of H⁺ donors was formulated in phosphate buffer to start the reaction. Then probe was submerged in sample, occasionally stirred gently and measured value (in %) was recorded once the reading was stabilized. End point reading was noted after 20 min (Tuitemwong *et al.*, 1994).

One oxyrase unit is defined as ability of integrated enzyme system in sterile cytoplasmic membrane fragments of *E. coli* to reduce 1% dissolved oxygen per mL of solution in one minute at 57°C, pH 8.5. Following equation was derived from Wongjaroen *et al.* (2008), and

used to calculate oxyrase activity (OA):

$$\text{OA (U/mL/min)} = \left(\frac{\% \text{Reduction in DO} \times \frac{1}{\text{Reaction volume (mL)}}}{\text{Time (minutes)}} \right)$$

Molecular confirmation of best E. coli isolate with maximum oxyrase activity

Ribotyping of the strain was performed for molecular confirmation. Extraction of genomic DNA of *E. coli* was performed with Thermo Scientific® GeneJET Genomic DNA (gDNA) Purification Kit# K0721 by using manufacturer's protocol. Conventional PCR was set up with specific universal primers for *E. coli*: 27F and 1492R (Frank *et al.*, 2008). Total PCR reaction mixture (25 µL) comprised: 12.5 µL Thermo Scientific® PCR Master Mix (2X) # K0171+0.5 µL of 10 µM each primer+2 µL *E. coli* gDNA, and rest of final volume was raised with deionized water.

PCR was set up in thermocycler (Biometra®) by initial denaturation at 94°C for 5 min followed by 30 cycles, each of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec and extension at 72°C for 2 min. Final extension was done at 7°C for 10 min. PCR product was purified with Thermo Scientific® GeneJET Gel Extraction and DNA Cleanup Micro Kit#K0831 by using manufacturer's protocol. Purified PCR product was run on 1% agarose gel (EtBr added in 0.5 µg/mL final concentrations). Thermo Scientific® GeneRuler DNA Ladder Mix, ready-to-use, # SM0334 (100-10,000 bp) was used as standard. BIO-RAD® agarose gel electrophoresis horizontal apparatus was used. Gels were allowed to run at 100V for 35 min.

PCR cleaned product containing amplified 16SrRNA gene from *E. coli* isolate was sent to Applied Biosciences International, Malaysia for sequencing. The 16SrRNA sequence was then aligned with representative 16S rRNA sequences belonging to related taxa with Clustal Omega online tool of EMBL-EBI (McWilliam *et al.*, 2013). By using neighbor-joining method (Saitou and Nei, 1987) and Jukes and Cantor model (Jukes and Cantor, 1969) of software MEGA 6.0 (Tamura *et al.*, 2013), phylogenetic tree was generated.

Use of Avestin Emulsiflex-C3® for preparation of oxyrase containing cytoplasmic membrane fragments

E. coli inoculum (30mL) was raised overnight in LB broth medium. Modified RF medium consists of peptone, 3.0 g/L; yeast extract, 2.5 g/L; KH₂PO₄, 2.5 g/L; MgSO₄·7H₂O, 0.2 g/L; glucose, 15.0 g/L and ammonium sulphate (NH₄(SO₄)₂), 2.0 g/L (Rattray and Fox, 1997) was formulated and dispensed separately in 1L volume in three 2.5L Thomson™ Ultra Yield™ flasks. Flask's necks were

covered by aluminium foil and sterilized by autoclaving. 10mL inoculum was aseptically added to each flask, and incubated for 16 h at 37°C, 180 rpm in New Brunswick Scientific™ Innova® 44 Incubator Shaker.

Culture broth was centrifuged at 4000rpm for 20 min at 4°C to pellet the cells. Cell pellet (1.0 g) was resuspended in 3mL of buffer (50mM Tris pH 8.0 + 300mM NaCl) by vortexing. EDTA (1mM final concentration) + 200µL benzoase from 2mg/mL stock in 50% glycerol + PMSF (phenylmethylsulfonyl fluoride) in final concentration of 0.01% was added to resuspended pellet and mixed by pipetting.

Resuspended cell pellet and buffer bottles were preceded on crushed ice for cell lysis by using Avestin Emulsiflex-C3® homogenizer. Cell lysis was performed according to standard protocol (Tong, 2011) and was harvested in a clean bottle placed on crushed ice. MgCl₂ (10mM final concentration) was added later on after cell disruption, and mixed by pipetting.

Disrupted/lysed cell sample was centrifuged at 17000rpm, 4°C for 30 min in Beckman fixed angle JA-17 rotor using Beckman Coulter® high speed floor centrifuge machine to pellet cell debris. Supernatant with membrane fragments were harvested and sterilized by using Corning® 500mL vacuum filter system, 0.22µm pore, 33.2cm² sterile polyethersulfone (PES) membrane for further analysis (Standard Operating Procedures v2.4 developed by Protein Expression Technology Center, Paul D. Boyer Hall UC Los Angeles DOE).

Characterization of oxyrase activity of emulsiflex prepared purified membrane fragments from E. coli

Effect of time (0-150 seconds) on oxygen scavenging potential of Emulsiflex mediated membrane preparation was evaluated started with 100% oxygen saturation. Characterization of emulsiflex mediated prepared oxyrase activity was also performed at 57°C and pH 8.5 as optimized in our previous work (Ahmad *et al.*, 2017) in presence of various concentrations (0-50mM) of Formate, Lactate, α-glycerophosphate and Succinate as H⁺ donors/ substrate.

Utilization of Avestin Emulsiflex-C3® prepared E. coli oxyrase containing cytoplasmic membrane fragments for improved cultivation of anaerobic bacteria

Pure and identified strains of four anaerobic test bacteria: *Anaerobaculum hydrogeniformans* strain OS1, *Akkermansia muciniphila*, *Bilophila wadsworthia* and *Roseburia intestinalis* were taken from culture stock of Department of Microbiology, Immunology and Molecular Genetics, University of California Los Angeles. Anaerobic cultivation of these strains for *in vitro* evaluation of

oxyrase by using Hungate Technique were setup according to protocols originally described by [Hungate \(1969\)](#) and [Bryant \(1972\)](#). Scheme for anaerobic media composition was provided by Prof. Dr. Robert P. Gunsalus (MIMG, UCLA). Detailed media composition for all four anaerobic bacteria used is provided in supplementary material file.

In one set of three tubes (for triplicate readings), 0.1mL of 2.5% Na₂S-Cys-HCl stock was aseptically added. In other set of three tubes (for triplicate readings), 0.6mL of oxyrase buffer reagent-without methylene blue ([Ahmad et al., 2017](#)) + 0.4mL of oxyrase preparation was aseptically added. Experimental/culture tubes were inoculated with 2% inoculum (0.6 OD_{600nm}). Uninoculated tubes for both Na₂S-Cys-HCl and oxyrase (as reducing agents) were separately set as blank for spectrophotometric analysis of growth. Biochrom® WPA CO8000™ Cell density meter, with test tube sample containers, was used for analysis of OD values at 600nm. Prepared media were autoclaved at 121°C (liquid cycle) for 30 min with slow exhaust cycle.

Determination of kinetic parameters of oxyrase

Double reciprocal Lineweaver-Burl plot was used to estimate K_m (Michaelis-Menten constant) and V_{max} (maximum reaction velocity) values of oxyrase for all substrates as described by [Lineweaver and Burk \(1934\)](#). Graph was plotted between reciprocal of V (reaction velocity, calculated according to equation A) and reciprocal of used substrates. Equation of straight line from plot was referred to Lineweaver-Burk equation (Equation B) to calculate kinetic parameters ([Bennett and Frieden, 1969](#)). Equations C and D were used to calculate V_{max} and K_m values.

$$V = \frac{\text{Initial DO } (\mu\text{M/L}) \text{ at } t_0 - \text{Final DO } (\mu\text{M/L}) \text{ at } t_1}{\Delta t} \dots\dots (A)$$

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \left(\frac{1}{S} \right) + \frac{1}{V_{\max}} \dots\dots (B)$$

$$y = mx + c \dots\dots (\text{Equation of straight line})$$

Whereas,

$$m = \text{slope of straight line} = \frac{K_m}{V_{\max}} \text{ and } c = y - \text{intercept}$$

$$V_{\max} = \frac{1}{c} \dots\dots (\text{Equation C})$$

$$K_m = V_{\max} \times \text{slope} \dots\dots (\text{Equation D})$$

Data analysis

For processing the data, statistical methods given by [Snedecor and Cochran \(1980\)](#) was adopted by using Costat Computer software. Experimental means were compared by Completely Randomized One Way ANOVA (Analysis of Variance). Duncan's Multiple Range Test was used for

this purpose with significance level of 5%.

RESULTS AND DISCUSSION

Present study was conducted for utilization of *E. coli* cytoplasmic membrane fragments' electron transport chain composite enzyme system hereby referred as oxyrase. This enzyme system is responsible for reduction in dissolved oxygen from the medium it has been employed and is very suitable for generation of microanaerobic environments for cultivation of anaerobic microorganisms. In our best knowledge, present work is first of its kind in which Emulsiflex is used for preparation of oxyrase, and Hungate technique was employed for evaluation of its antioxidant potential.

For the purpose of isolation of *E. coli*, poultry intestine samples because of its heavy colonization with the bacterium. Out of 30 intact poultry intestine samples; 08 samples were collected from site 1 (Anarkali) resulted in 28 isolates of *E. coli* (EC1-EC28); 19 isolates (EC29-EC47) and 41 isolates (EC48-88) were found in 7 samples from site 2 (Rehmanpura) and 15 samples from site 2 (Ichhra), respectively. *E. coli* isolate showed differential appearance of characteristic metallic sheen colonies of *E. coli* on EMB agar medium as reported by [Cheesbrough \(2006\)](#). This result is in accordance to [Jin et al. \(1997\)](#) as they have also reported *E. coli* isolation with its abundant distribution in duodenum and jeju-ileum of chicken. Frequent isolation of large number of *E. coli* strains from poultry intestines in present work is in accordance with [Carson et al. \(2001\)](#). They showed similar kind of results reported for identification of abundant fecal *E. coli* isolates from humans and animals. Furthermore, they also reported heavy inhabitation of *E. coli* in poultry intestinal tracts.

These 88 (numbered in series as EC1-EC88) were pink red Gram negative straight rods. They gave negative oxidase, citrate utilization and gelatin hydrolysis test and positive catalase carbohydrate fermentation and methyl red tests. These results in accordance to [Holt et al. \(1994\)](#) and [Cheesbrough \(2006\)](#) inferred their identification as *E. coli*. EC4 was the best strain in terms of oxygen reduction ability, with activity of 0.41±0.008 U/mL/min which dissipated 41% of dissolved oxygen at standard defined conditions.

Frequency of active oxyrase by probe method suggested 76 isolates were positive for oxygen reduction potential. 37% of positive isolates showed activity range from 0.25-0.34 U/mL/min, 20% isolates with 0.15-0.24 U/mL/min, 22% isolates with 0.05-0.14 U/mL/min and 20% isolates' activity ranged ≤0.04U/mL/min. This distribution of frequency in activity is in accordance along with variation to [Wongjaroen et al. \(2008\)](#). Variation in results

of present and their study is due to difference in enzyme assay reaction mixture; and they also did not reported source habitat from where *E. coli* was isolated.

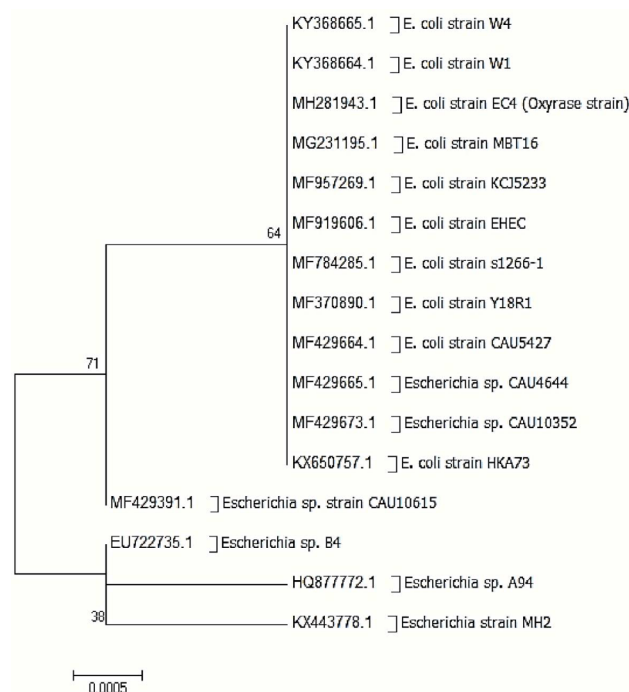


Fig. 1. Phylogenetic tree showing evolutionary relationship between isolated *E. coli* strain EC4 (oxyrase strain) and some reference strains. The number at the branch nodes indicates bootstrap values (%) based on 1000 replication. The scale bar represents 0.0005 nucleotide substitutes per position.

On the basis of screening results, *E. coli* oxyrase strain (EC4) was selected and further preceded for confirmation by ribotyping. PCR product with 1.5kB size 16SrRNA gene sequence was used for molecular confirmation of *E. coli* oxyrase strain. PCR with universal primers: 27F and 1492R, specific for *E. coli* was employed and agarose gel electrophoresis results showed specific amplification of gene size 1.5kB. This result is in accordance with Frank *et al.* (2008). Sequencing results were further analyzed by using online BLASTN 2.7.1+ tool of NCBI. Multiple sequence alignment was performed with online MUSCLE tool of EMBL-EBI. Phylogenetic tree constructed with MEGA 6.0 showed significant evolutionary relationship with 11 of *E. coli* strains with closest being *E. coli* strain W4 and *E. coli* strain KCJ5233 (Fig. 1). Carson *et al.* (2001) also reported confirmation of abundant *E. coli* by ribotyping, isolated from animal fecal samples. Ribotyping and Biochemical profiling confirmed EC4 strain as *E. coli* which was further used in rest of experimental work.

The sequence was submitted to GenBank of NCBI with accession no. MH281943.1 and can be accessed online at <https://www.ncbi.nlm.nih.gov/nuccore/MH281943>.

Characterization of these purified Emulsiflex prepared membrane fractions were performed for quantification of active oxyrase; which revealed 100% reduction in dissolved oxygen at 57°C, pH 8.5, in presence of 25mM lactate as H⁺ donor, after 1.5 min with activity of 20±0.49 U/mL and specific activity of 8.04 U/mg (Figs. 2, 3). Maximum activity of 13.33±0.19 U/mL/min was observed at 25mM lactate. Increase in proton donor concentration resulted increase in oxygen reduction potential of membrane fragments. But at higher concentrations (≥25mM lactate) stable trend in oxyrase activity was observed. Response of Emulsiflex prepared oxyrase membrane fragments towards all substrates in various concentrations was found same as of sonication prepared fragments as reported in our previous work (Ahmad *et al.*, 2017).

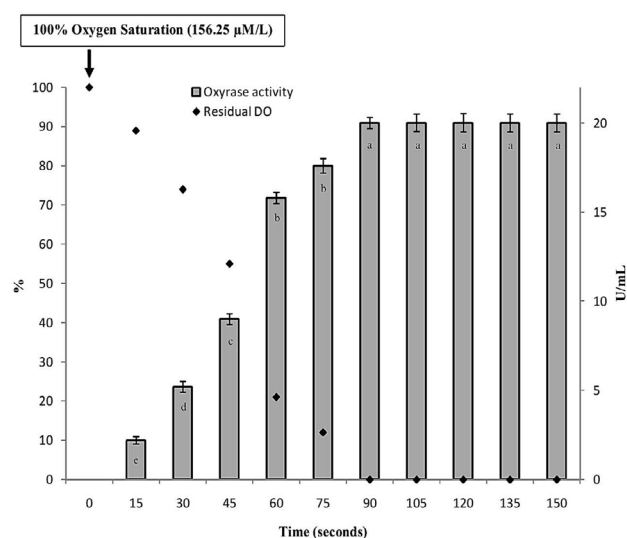


Fig. 2. Effect of time on oxyrase activity (emulsiflex mediated prepared membrane fragments); 57°C temperature, pH 8.5 and 25mM lactate as H⁺ donor. Y-error bars represents the standard deviation among the triplicates. Each value is an average of three replicates, data labels followed by different letters differs significantly at $\alpha=0.05$.

In presence of formate (25mM), 93.75% reduction in dissolved oxygen was observed, whereas α -glycerophosphate and succinate (25mM concentration) caused 81% and 70.5% reduction in DO, respectively. These results are in accordance to Tuitemwong *et al.* (1994) as they reported 100% depletion in oxygen in presence of lactate within 2 min. Therefore, this activity of oxyrase is 95% improved per unit time as compared to

sonication mediated cell lysis for oxyrase production from *E. coli* in classical Erlenmeyer shake flask experiments. This dramatic improvement of oxygen reducing potential of Emulsiflex prepared *E. coli* membrane fragments over sonication prepared is due to obvious efficiency of Emulsiflex to cause cell lysis and keeping integral enzyme system intact in membranes (Andrews and Asenjo, 1987).

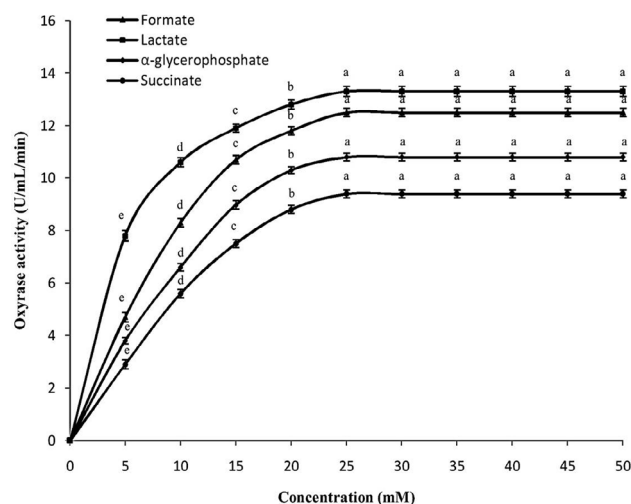


Fig. 3. Effect of initial concentration of H^+ donors on oxyrase activity; time 1.5 min, 57°C temperature, pH 8.5. Y-error bars represents the standard deviation among the triplicates. Each value is an average of three replicates, data labels followed by different letters differs significantly at $\alpha=0.05$.

Highly improved oxyrase efficiency of Emulsiflex prepared membrane fragments can also be justified due to use of THOMSON™ ULTRA YIELD™ shake flasks in present work. These findings are in accordance to Ukkonen *et al.* (2011) and Brodsky and Cronin (2006). Moreover, regulated and improved protein production by *E. coli* using Emulsiflex is also reported by Schlegel *et al.* (2013), Savage *et al.* (2007) and Hintz *et al.* (2001). But use of THOMSON™ ULTRA YIELD™ and Avestin Emulsiflex-C3® Homogenizer for production of oxyrase form *E. coli* is first of its kind in present study.

Downstreaming of oxyrase membrane fragments from *E. coli* in presence of buffer, EDTA, benzoase, and PMSF also played vital role in improved activity of oxyrase in present study. *E. coli* cell pellet was resuspended in buffer constituted by mixing 50mM Tris pH 8.0 and 300mM NaCl. This buffer provided buffered slight alkaline and salt environment to biological membranes which dissociates extrinsic peripheral membrane proteins by disrupting

hydrogen and electrostatic bonds. Membrane fragments constituted oxyrase activity are integral proteins extended in *E. coli* lipid bilayer. Therefore, the said buffer provided better physiological and biochemical environment to membrane preparations for better yield in Emulsiflex (Ohlendieck, 2004). EDTA is chelating agent and it chelated all Mg^{++} and Ca^{++} ions from outer LPS membrane in *E. coli*, therefore destabilized it and makes cell lysis easy to expose cytoplasmic membrane fragments better (EMBL protein purification protocol). Benzoase nuclease degraded all form of cellular DNA and RNA released from lysed cell. PMSF is a serine protease inhibitor and its addition in cell Lysate avoided protein loss by degradation (James, 1978). $MgCl_2$ (10mM) was added after cell lysis to neutralize further effect of any residual EDTA left in cell Lysate.

In vitro evaluation of oxyrase in cytoplasmic membrane fragments of *E. coli* strain EC4 was investigated for its active antioxidant by using the Hungate technique. In first experiment, effect of *E. coli* oxyrase and its comparison to Cys-HCl was evaluated on growth of a moderately thermophilic and anaerobic bacterium *Anaerobaculum hydrogeniformans* OS1. Bacterium reached maximum OD_{600} of 0.65 ± 0.016 with Cys-HCl, and 0.80 ± 0.017 with oxyrase as reducing agent at 55°C after 4 days of incubation (Fig. 4A). This is a clear evidence of efficient oxygen reducing ability of oxyrase comparative to Cys-HCl, as oxyrase supplemented culture tubes reached 19% more cell density as compared to oxyrase. It is also obvious that during late growth phase, *Anaerobaculum hydrogeniformans* OS1 cells maintained higher OD_{600} value of 0.59 ± 0.014 with oxyrase and 0.25 ± 0.018 at day 15 of incubation. This is due to slow reduction ability of Cys-HCl and its reaction with dissolved oxygen results in production of chemical residues that proved toxic for bacterial growth (Maune and Tanner, 2012).

Efficiency of *E. coli* oxyrase was evaluated with growth of strictly anaerobic bacterium *Akkermansia muciphila* in second experiment. After 18 h of incubation at 37°C , *Akkermansia muciphila* cells reached maximum OD_{600} of 2.10 ± 0.07 with oxyrase and OD_{600} of 1.66 ± 0.05 with Cys-HCl. As expected, oxyrase worked with efficiency of 21% improvement in cell growth compared to Cys-HCl. Whereas, Cys-HCl tubes supplemented culture tubes reached maximum cell density with OD_{600} 2.00 ± 0.05 after 27 h of incubation. Furthermore, it is also very obvious from growth curve (Fig. 4B) that in late growth phase from time 18-24 h of incubation, *Akkermansia muciphila* cells density remained stable with oxyrase. During the same time of growth with Cys-HCl, the OD_{600} value did rise from initial value 1.66 ± 0.05 to 2.00 ± 0.05 .

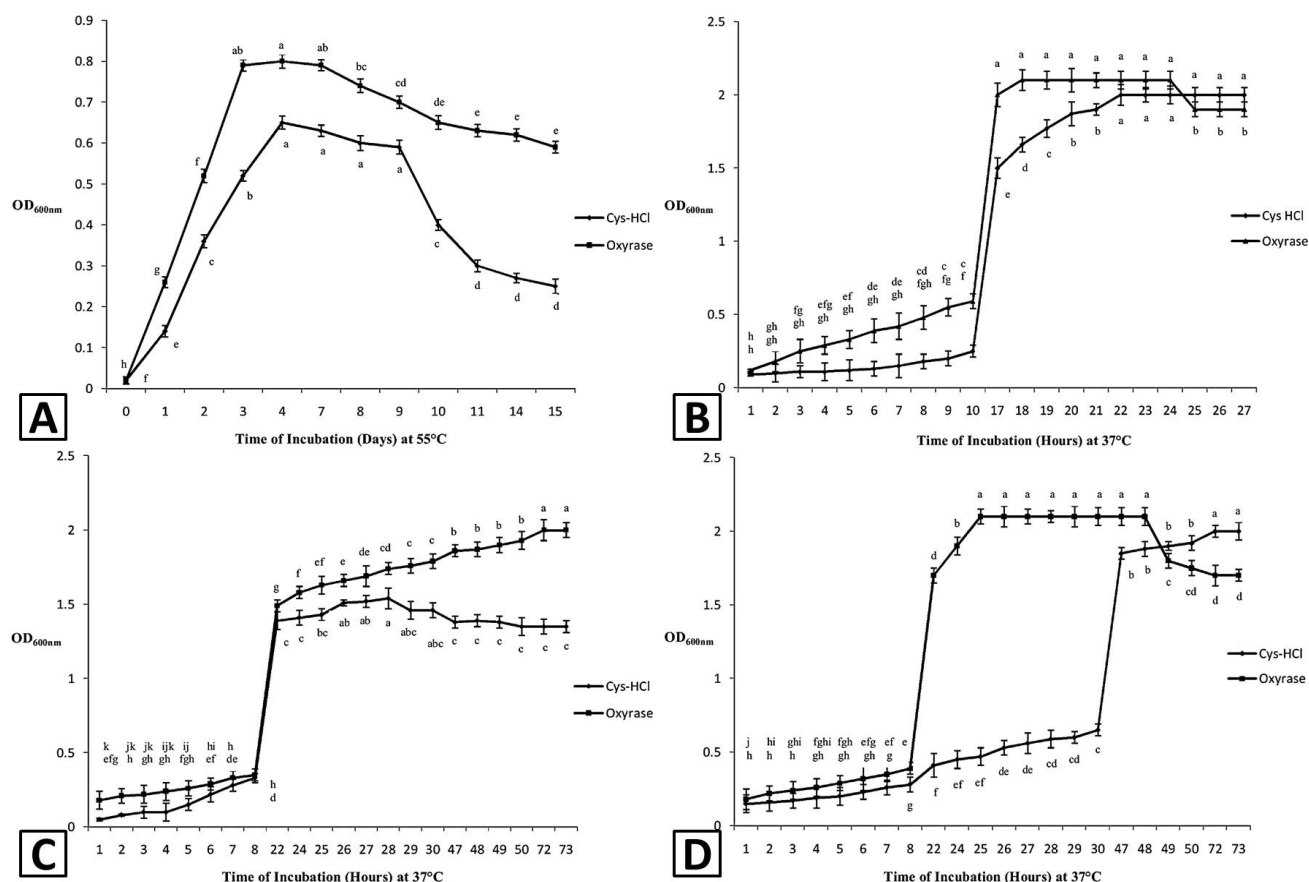


Fig. 4. Comparison and effect of Cys-HCl and *E. coli* oxyrase on growth pattern of *Anaerobaculum hydrogeniformans* OS1 (A), *Akkermansia muciniphila* (B), *Bilophila wadsworthia* (C) and *Roseburia intestinalis* (D). Y-error bars represents the standard deviation among the triplicates. Each value is an average of three replicates, data labels followed by different letters differs significantly at $\alpha=0.05$.

This observation clearly showed sustainable efficiency of oxyrase membrane fragments to reduce dissolved oxygen efficiently and quickly as compared to Cys-HCl. During last three hours of growth phase, cell density with oxyrase decreased to 1.90 ± 0.05 due to obvious reason of decline/death phase of growth curve initiated at that point. Use of oxyrase for growth of *Akkermansia muciniphila* and its comparison with Cys-HCl using Hungate technique is not reported before. However, Gehring *et al.* (2014) reported efficient recovery of another facultative anaerobic, mesophile bacterium *L. monocytogenes* from food samples, with commercially available EC-oxyrase.

A third experiment was conducted to evaluate efficiency of *E. coli* oxyrase on an obligate anaerobic clinically relevant bacterium *Bilophila wadsworthia*. This bacterium is reported to cause intra-abdominal abscesses (Feng *et al.*, 2017; Baron *et al.*, 1992). During initial growth phase of 8 hours, cell density of *Bilophila wadsworthia* remained almost same: 0.33 ± 0.03 with

Cys-HCl and 0.35 ± 0.04 with *E. coli* oxyrase as reducing agents. Bacterium reached maximum OD₆₀₀ of 2.00 ± 0.05 in presence of oxyrase and 1.35 ± 0.04 with Cys-HCl after 73 h of incubation, indicating 33% improvement in cell density with oxyrase (Fig. 4C). Another significant difference is that after 28 h of incubation, *Bilophila wadsworthia* cell density was 1.54 ± 0.07 with Cys-HCl, which later on entered in decline phase and cell density started decreasing. This is due to generation of toxic chemical residues by Cys-HCl in culture tubes during late growth phase. However, with oxyrase the cell density not only remained stable during this phase of growth but a rise in OD₆₀₀ of *Bilophila wadsworthia* cells was observed.

Use of Hungate technique with *E. coli* oxyrase for *Bilophila wadsworthia* is not reported before and it's first of its kind in present study. Results showed presence of oxyrase supported growth of *Akkermansia muciniphila* much better as compared to Cys-HCl. Growth was improved by 20% in presence of oxyrase and maximum

OD_{600nm} of 2.1 ± 0.07 was achieved in tubes with oxyrase after 18 h of incubation showing its efficiency to remove dissolved oxygen (Fig. 4D). However, Wongjaroen *et al.* (2008) evaluated membrane fragments extracted from *E. coli* on growth enteric, microaerophilic pathogenic bacterium *Campylobacter jejuni*. They also reported improved cultivation of *Campylobacter jejuni* in increased amount of *E. coli* membrane fragments in Mueller Hinton Broth medium without chemical reducing agents.

Figure 5 shows Lineweaver-Burk plot for Emulsiflex prepared oxyrase characterized at standard conditions. Present study is first of its kind for determination of kinetic parameters of oxyrase. Minimum K_m value of $4.75 \times 10^{-3} \text{ M}^{-1}$ was observed with lactate as H^+ /substrate which defined maximum affinity of oxyrase membrane fragments with lactate for oxygen reducing potential. Second best affinity of membrane fragments was found for formate with K_m value of $1.34 \times 10^{-2} \text{ M}^{-1}$. α -glycerophosphate and succinate showed weak affinities out of all used substrates with K_m values of $1.76 \times 10^{-2} \text{ M}^{-1}$ and $2.13 \times 10^{-2} \text{ M}^{-1}$, respectively. Detail of kinetic parameters is shown in Table I.

Table I.- Comparative kinetic parameters of Emulsiflex prepared *E. coli* strain EC4 derived oxyrase

Substrate	V_{\max}	$K_m (\text{M}^{-1})$	V_{\max} / K_m
Formate	142.85	1.34×10^{-2}	1.06×10^4
Lactate	125	4.75×10^{-3}	2.63×10^4
α -glycerophosphate	142.85	1.76×10^{-2}	8.13×10^3
Succinate	125	2.13×10^{-2}	5.88×10^3

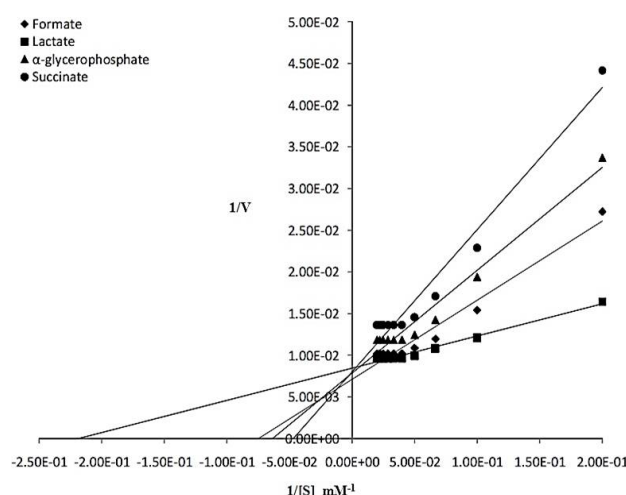


Fig. 5. Double reciprocal Lineweaver-Burk plot between inverse of V and inverse of different concentrations of various substrates for emulsiflex prepared oxyrase membrane fragments at 57°C, pH 8.5 and time 1.5 min.

CONCLUSION

Oxyrase is a composite enzyme in *Escherichia coli* cytoplasmic membrane fragments which comprises synergistic activity of dehydrogenases and terminal reductases/oxidases in *E. coli* membranes as part of its electron transport chain. Oxyrase bearing membrane fragments were prepared Avestin Emulsiflex-C3® mediated lysis of local *E. coli* strain EC4 cells, isolated from poultry intestinal sample. Avestin Emulsiflex-C3® is used for first time for preparation of oxyrase in present work. These membrane fractions showed enhanced oxyrase activity with $13.33 \pm 0.19 \text{ U/mL/min}$ and specific activity of 8.0 U/mg which caused 100% reduction in dissolved oxygen at 57°C, pH 8.5 after 1.5 min. Kinetic parameters also supported evidenced of strong substrate affinity by Emulsiflex mediated oxyrase membrane preparation. Hungate technique was employed for anaerobic cultivation of organisms and antioxidant potential of oxyrase was compared with commonly used dissolved oxygen scavenger Cys-HCl. Oxyrase outcompeted Cys-HCl and efficiently supported improved growth rates of tested anaerobic strains. This strategy for production and evaluation of oxyrase is hereby first time used in present work. Findings of present research work will prove bench mark for developing better facility of cultivation of anaerobic bacteria either for diagnostics or research purpose. As a specialized facility for cultivation of anaerobes is not available in Pakistan, use of oxyrase will replace inefficient and cumbersome approaches to achieve anaerobiosis and to use air tight anaerobic chambers to separately support growth of anaerobic bacterium.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: <http://dx.doi.org/10.17582/journal.pjz/2019.51.3.835.834>

Statement of conflict of interest

The authors declare no conflict of interest.

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