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EXPLORATION AND SCREENING OF OXYGEN REDUCING POTENTIAL IN CYTOPLASMIC MEMBRANE FRAGMENTS OF *ESCHERICHIA COLI* AND *SALMONELLA SP.*

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ABSTRACT

Partially purified cytoplasmic membrane fragments derived from 24 hours old cultures of poultry isolates of *Escherichia coli* and *Salmonella sp.* were screened for presence of active oxyrase. At 37°C and pH 7.5, membrane fragments of *Escherichia coli* and *Salmonella sp.* reduced 40% and 35% dissolved oxygen respectively in presence of 10mM lactate after 20 minutes. This oxygen reducing ability of each bacterial fragment is due to the active oxyrase present and its ability to dissipate oxygen was increased to 45% for *Escherichia coli* and 48% for *Salmonella sp.* at pH 8.5. After further characterization, maximum activity of 1.62U/ml of oxyrase (that depleted 65% dissolved oxygen) for *Escherichia coli* and 1.42U/ml (that depleted 57% dissolved oxygen) for *Salmonella sp.* was observed at 57°C, pH 8.5 after 20 minutes.

Keywords: *Oxyrase, Methylene blue, Plasmoptysis, Dissolved-oxygen probe.*

INTRODUCTION

Many bacteria contain enzyme systems in cytoplasmic-membranes that efficiently reduce dissolved oxygen to water. These enzymes and related factors, usually referred as electron-transport systems, are significant components of the mechanisms through which high energy phosphate bonds are produced by living cells (Adler *et al.* 1981). Moller and Hederstedt (2006) reviewed the importance of two membrane bounded thiol-disulfide oxidoreductases in efficient production of heat-resistant endospores in *B. subtilis*. Applications of membrane-embedded enzymes in several Redox reactions have been reported, that require O_2 as one of the substrates. Food microbiologists can utilize such membrane bound enzymes, as antioxidants, to reduce molecular oxygen in processed foods, due to its oxygen scavenger property (Palmer & Bonner, 2007). Now days, in many industrial processes the use of harsh chemicals have been gradually replaced by enzymes for the purpose of sustainable and environmental friendly bioprocessing (Malathu *et al.* 2008).

Adler (1990) has reported presence of active oxygen reducing membrane fragments in several bacterial genera. Ho *et al.* (2003) and Petersen & Bradford (2005) evaluated the use of sterile membrane fragments of *E. coli*, commercially available as Oxyrase, on growth of several species of anaerobic bacteria without using other means of removing O_2. Other current methods for cultivation of anaerobic microorganisms rely on the use of airtight jars, bags and anaerobic chambers, from which the oxygen is eliminated by employing inorganic catalysts or gas flushing. Practical implementation of these methods can be laborious. Furthermore, besides removal of oxygen from culturing glassware/chambers it is also needed to be done in culture media, which is conventionally done by addition of reducing agents like Sodium Thioglycollate. In contrast to these agents and devices, it is anticipated that the addition of oxyrase containing membrane fractions directly to the media would remove oxygen rapidly and efficiently, conferring the process to be non-toxic (Crow *et al.* 1985).

Present study was carried out to screen the presence of oxyrase (oxygen reducing enzyme) in partially purified cytoplasmic membrane fragments of *Escherichia coli* and *Salmonella sp.* isolated from poultry enteric tract. Moreover, it was also aimed to characterize the optimum in vitro working conditions of oxyrase.

MATERIALS AND METHODS

Organisms: *Escherichia coli* and *Salmonella sp.* were isolated from chicken droppings and intact intestines collected from 10 different butcher shops in Lahore. Chicken droppings were then 10^{-1} diluted in sterile PBS (pH 7.4) whereas, intact chicken intestines were aseptically homogenized in sterile PBS (pH 7.4) and homogenate was diluted to 1:20 in sterile normal saline solution accordingly. 0.1ml of each diluted sample was uniformly distributed and inoculated separately on Eosin Methylene Blue (EMB) agar medium, Salmonella-Shigella (SS) agar medium and MacConkey agar medium plates which were incubated at 37°C for 24hrs (James & Sherman, 2007). After incubation, above mentioned selective and differential media were screened for presence of characteristic colonies for required bacteria,

which were further identified by using microscopy, colonial and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994) and Cheesbrough (2006). Confirmed bacterial isolates were pure-cultured on sterile nutrient agar plates by quadrant streaking, and maintained at 4°C for a week.

Up-streaming Process: Loop-full of pure, identified *Salmonella* and *E. coli* cultures were separately inoculated in 25ml nutrient broth medium in a 250ml Erlenmeyer Shake flasks, and kept for 24 hrs in shaking incubator (VS-8480, Vision Scientific CO. LTD, Korea) at 37°C, 180rpm in order to obtain OD_{600nm} at 2.00 measured against sterile, un-inoculated nutrient broth (Sivashanmugam *et al.* 2009).

Downstreaming Process: Bacterial broth cultures were aseptically transferred to sterile falcon tubes, and then centrifuged at 7000g for 15 minutes in the Centrifuge (HT Compan, UK) at 4ºC. After 15 minutes, the pellet of bacterial cells was obtained at the bottom of the falcon tubes. The media in supernatant was decanted aseptically in a beaker and pellet was suspended by vortexing in 15ml sterile PBS (pH 7.4). This re-suspended pellet was again centrifuged at 7000g for 15 minutes. These steps were repeated for three times until washed suspensions of *E. coli* and *Salmonella sp.* were obtained. Washed cell suspension was stored at 4ºC. Turbidity of cell suspension was maintained to OD_{600nm} of 1.6. For cell lysis, pellet of washed bacterial cells were suspended in 15ml of sterile Hypotonic Lysis Buffer (10 ml of 40mM of HEPES mixed with 2.5ml of 40mM of NaCl and 5.0ml of 10mM of EDTA, final volume raised to 250ml with distilled water) and placed for 15 minutes that allows plasmoptysis of bacterial cells which will ease the cell lysis process in sonication procedure.

Plasmpotysed bacterial cell suspension was placed on crushed ice and subjected to six cycles, with 80 seconds interval, of sonication at frequency 20 kHz. Sonicated product was smeared on glass slide and microscopically observed for confirmation of proper cell lysis. Cell sonicate was centrifuged and pellet containing crude membrane fractions was suspended in 0.01M HEPES Buffer (pH 7.5) to a solid content of 40mg/ml (Suslick & Flannigan, 2008). Membrane-bound enzyme fractions were twice filter-sterilized through syringe filter of 0.45µm pore size. All samples were placed at -10°C in refrigerator until use. Sterility of *E. coli* and *Salmonella sp.* membrane fractions was tested by adding 1ml of each fraction to LB medium (pH 7.0), incubated at 37°C for 48hrs (Tuitemwong *et al.* 1994).

Oxyrase Bioassay: Methylene blue based assay was performed for screening of active oxyrase presence in sterile bacterial membrane fragments as qualitative method. Three millilitre of methylene blue assay reagent (100 ml of 40 mM Phosphate Buffer was made at pH 8.4 with 20mM of dl-lactate and 1 µg/ml methylene blue solution was added into the buffer) was added into each of three screw-cap test tubes and warmed in water bath at 37°C. 2 ml of membrane fragments was added in two test tubes (one for each bacterium) and one was labelled as negative control. The tube contents were not mixed and maintained upright without disturbance. The screw caps were affixed and these were incubated at 37°C for twenty minutes. Tubes were kept under continuous observation during incubation time for gradual discoloration of Methylene blue's dark-purple colour (Oxyrase, Inc. 2015).

Quantification of oxyrase units was carried out with dissolved oxygen (DO) meter using modified method of Tuitemwong *et al.* (1994). 10 ml of 20mM lactate was added in 40 ml of sterile distilled H_2O contained in 250ml sterile conical flask, and incubated at 37°C for 20 minutes in a water bath. After incubation, Clark's polarographic construction type probe was dipped in flask was left undisturbed for about 15 minutes till the reading of the meter became stable. The initial oxygen concentration in distilled water was the control for 100% oxygen saturation. Two millilitre of each membrane fractions were added to 8 ml of phosphate buffer (pH 7.5). The suspension was placed in the 37°C incubator for 3 minutes. Then the reaction was started with the addition of 10 ml of the 10mM substrate and kept on incubation at 37°C; rate of oxygen depletion at standard conditions were monitored every 10 second to 25 minutes. Difference in the readings of the experimental from that of the control gave the hint about the amount of oxygen that was reduced by oxyrase enzyme system, present in the membranes of *E. coli* and *Salmonella sp*.

One unit of Oxyrase activity is defined as ability of sterile bacterial membrane fragments to reduce 1% dissolved oxygen per minute at 37°C, pH 8.5 in phosphate buffer with 10mM lactate as a substrate.

RESULTS AND DISCUSSION

Seven different isolates were observed on EMB, SS and MacConkey agar medium. Out of total seven isolates, two were identified as *E. coli* and *Salmonella sp.* on basis of microscopic observation of gram-stained smear, colonial and biochemical characteristics (Table 1).

Two isolates (IIB-*EC*-1 and IIB-*SAL*-3) revealed the presence of active oxyrase in sterile membrane fragments. Test tube containing *Escherichia coli* (IIB-*EC*-1) membrane fragments showed discoloration of methylene blue after 15 minutes of incubation, whereas membrane fragments of *Salmonella sp.* (IIB-*SAL*-3) caused discoloration in 20 minutes. Control test tube showed no discoloration. After clearing has reached the top, tubes with membrane fragments were gently inverted two times and buffer in the tubes turned blue again as oxygen in the head space has dissolved back into the

buffer. Within approximately the same time, the active Oxyrase in membrane fragments turned the solution clear once more. This result is in accordance to Adler (1990) as he described that various bacterial species like *Escherichia coli*, *Pseudomonas aeruginosa*, *Gluconobacter oxydans*, *Azotobacter vinlandii*, *Mycobacterium phlei* and *Salmonella typhimurium* have been used as a source for active oxyrase membrane fragments.

Quantitative results revealed that *E. coli* membrane fragments dissipated 40% dissolved oxygen in 20 minutes at standard conditions whereas, membrane fragments derived from *Salmonella sp.* actively reduced 35% dissolved oxygen in 20 minutes. Partially purified membrane fragments of *E. coli* and *Salmonella sp.* contain 1U/ml and 0.875U/ml of active oxyrase respectively at standard conditions.

Fig.1 and Fig.2 demonstrates the effect of proton donor on the activity of oxyrase. It was observed that for both *E. coli* and *Salmonella sp.* lactate proved best proton donor or substrate for activity of oxyrase as in its presence, 40±3.13% and 35±3.08% of dissolved oxygen was reduced respectively in 20 minutes. This result is contrary to the result reported by (Tuitemwong *et al.* 1994) as their results showed formate as best hydrogen donor for oxygen reducing activity of *E. coli* (with maximum of oxygen depleted after 1 min), lactate as best hydrogen donor for *Gluconobacter oxydans* (with maximum dissolved oxygen depleted in 2 minutes) and lactate & α-glycerophosphate as best hydrogen donors for *Acetobacter xylinum* (maximum oxygen dissolved oxygen depleted after 5 minutes). This contradiction in results of both works is may be due to the source of bacterial isolation and different microorganisms, as in our study both bacterial strains were isolated from enteric tract of poultry that might influence the physiological requirements of the enzyme, besides this no significant difference in ability of oxygen reduction with lactate and formate as proton donors for *E. coli* is evident.

The trend of substrate activity in both works is same that justifies the results of present work. But the time required for dissipation of oxygen is significantly different (higher in present study) for our strains as the membrane fragments used by Tuitemwong *et al.* (1994) were highly purified. In present study, Formate was the second best substrate for oxyrase activity as with this proton donor, *E. coli* and *Salmonella sp.* membrane fragments reduced 36±3.04% and 27±2.31% dissolved oxygen respectively.

Present work shows that α-glycerophosphate was third best and succinate was fourth best hydrogen donor for oxyrase activity of both bacterial membrane fragments (maximum oxygen reduced by *E. coli* was 18±2.98% and 19±3.98% by *Salmonella sp.* with α-glycerophosphate as substrate; whereas maximum of 12±2.98% and 14±2.38% oxygen was reduced by *E. coli* and *Salmonella sp.* membrane fragments with succinate as hydrogen donor for oxyrase activity respectively after 20 minutes for each reaction).

Tuitemwong *et al.* (1994) also reported same types of result that α-glycerophosphate and succinate did not reacted well with oxyrase of *Escherichia coli* and for *Gluconobacter oxydans* respectively. Whereas very little work has been reported for use of *Salmonella sp.* for characterization of oxyrase activity which has been explored and reported for the first time in this research work.

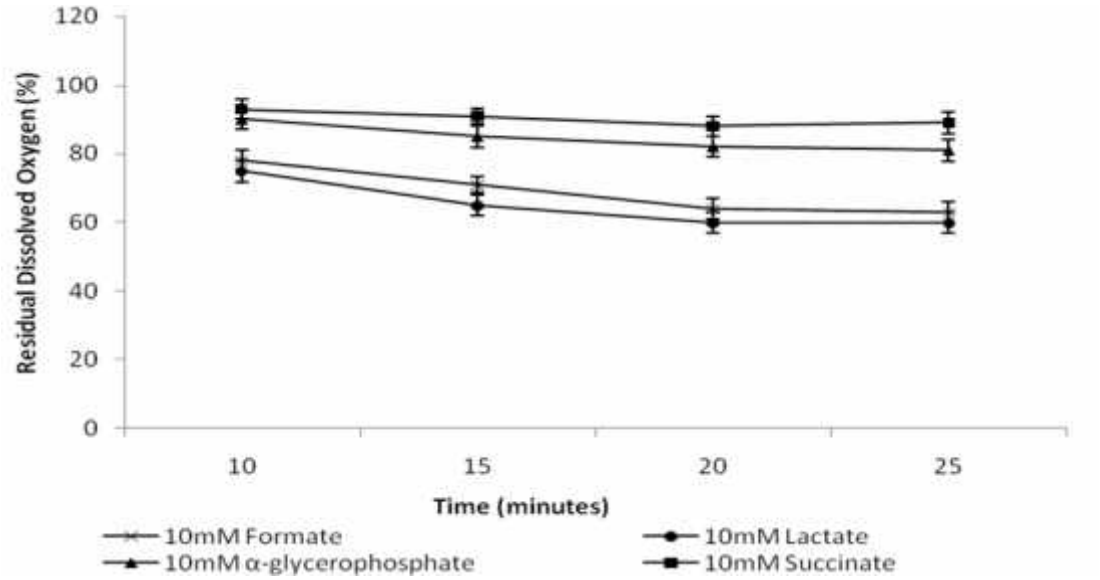

Figure 1. Effect of different substrates/H^+ Donor on oxyrase activity of *Escherichia coli* (pH 7.5, Temperature 37°C)

Table 1: Identification of bacterial isolates from poultry samples on the basis of growth, cultural, microscopic and biochemical characteristics

	Isolate No.1	Isolate No. 2	Isolate No. 3	Isolate No. 4	Isolate No. 5	Isolate No. 6	Isolate No. 7
Growth/Colonial characteristics on EMB agar medium	+++/dark-violet, greenish metallic sheen colonies with black center	+++/large, pink colonies with blue center	+++/large, colorless colonies	+/colorless colonies	++/colorless, cotton like	+/dark-violet colonies with dot or point like appearance	+++/large colorless colonies
Growth/Colonial characteristics on SS agar medium	+/pink, bile precipitated colonies, H_2S negative	+/cream-pink colonies, H_2S negative	+++/colorless, black centred colonies, H_2S positive	–	–	–	+++/colorless colonies without black centers, H_2S negative
Growth/Colonial characteristics on MacConkey agar medium	+++/pink-red, bile precipitated colonies	+/colorless to pink colonies	+++/colorless colonies	–	–	+/colorless to pink colonies	+++/large colorless colonies
Gram stain reaction	pink, straight rods with peritrichous flagella	pink, straight rods with peritrichous flagella	pink, straight rods and most cells with peritrichous flagella	purple/violet, spherical cells, arranged in pairs and in irregular clusters	Gram +ve, large ovoid, budding cells	purple/violet, spherical cells arranged in short chains	pink, straight rods without flagella
Oxidase Test	–	–	–	–	ND	–	–
Catalase Test	+	+	+	–	ND	–	+
Citrate utilization Test	–	+	+	+	ND	+	–
Urea hydrolysis Test	–	–	–	ND	ND	ND	–
Acid from Lactose	+	+	–	+	ND	+	–
Acid from Maltose	+	+	+	ND	ND	ND	–
Gelatin hydrolysis Test	–	–	–	ND	ND	ND	–
Methyl red Test	+	–	+	ND	ND	ND	+
Bacterial strain identified on the basis of above mentioned tests	*Escherichia coli*	*Enterobacter sp.*	*Salmonella sp.*	*Staphylococcus sp.*	*Candida sp.*	*Enterococcus sp.*	*Shigella sp.*
Active oxyrase in membrane fragments	+	–	+	–	–	–	–
Isolate Code	(IIB-*EC*-1)	(IIB-*ENTB*-2)	(IIB-*SAL*-3)	(IIB-*STAPH*-4)	(IIB-*CAND*-5)	(IIB-*ENTC*-6)	(IIB-*SHIG*-7)

'v' = variable reaction; ND = Test not determined; '+' for biochemical tests = positive; '-' for biochemical tests = negative; '+++' for growth = good-luxuriant growth; '++' for growth = Fair-good growth; '-' for growth = No growth

Effect of pH on oxyrase activity of *Escherichia coli* and *Salmonella sp*. revealed, in phosphate buffer of pH 8.5, maximum of 45±2.98% and 48±3.08% dissolved oxygen was reduced respectively (Fig. 3 & Fig. 4). Present study shows oxygen reducing ability in *Escherichia coli* membrane fragments at pH5.5: 8±3.23%; pH 6.5: 25±2.28%; pH 7.5: 39±3.1%; pH 9.5: 10±3.2%, whereas for *Salmonella sp.* at pH 5.5: 7±2.95%; pH6.5: 20±3.1%; pH 7.5: 37±2.8% and pH 9.5: 8±3.2%. Activity of oxyrase in both bacterial fragments was low at pH 5.5, but at pH 8.5 approximately 6 folds increase in oxygen reducing activity was observed for *Escherichia coli* and 7 folds increase for *Salmonella sp.* This indicates excellent potential of this enzyme for use as preservative in alkaline foods. This behaviour of our enzyme at different pH is in agreement of results reported by Tuitemwong *et al.* (1994) and also by Oxyrase Inc. for EC-Oxyrase®. However for *Salmonella sp.* it is not reported before.

Fig. 5 describes the effect of temperature on activity of oxyrase, maximum activity of oxyrase (1.62±0.018 U/ml) for *E. coli* was observed at 57°C which dissipated 65% of dissolved oxygen, this indicates a hint of thermostable behaviour of oxyrase; whereas there was an increasing trend in oxyrase activity with increase in temperature as: 27°C (0.77±0.042U/ml with 28% reduction in dissolved oxygen), at 37°C (1±0.014 U/ml with 40% reduction in dissolved oxygen), at 47°C (1.34±0.011 U/ml with 55% reduction in dissolved oxygen) and at 67°C (0.92±0.012 U/ml with 37% reduction in dissolved oxygen).

These results are in accordance with Tuitemwong *et al.* (1994) and commercial EC-Oxyrase® by Oxyrase, Inc. (2015). But these two sources have reported much more units of oxyrase from *E. coli* than present study because they have employed better purification process by ultracentrifugation whereas, in present work only partial purification of membrane fractions was performed. However, the trend for activity at different temperatures is same as reported earlier. Whereas, no literature was reported before for quantification of active oxyrase in membrane fragments of *Salmonella sp.*

Maximum activity of oxyrase (1.42±0.014 U/ml with 57% reduction in dissolved oxygen) was observed for *Salmonella sp*. at 57°C, whereas at 27°C (0.62±0.013 U/ml with 25% reduction in dissolved oxygen), at 37°C (0.87±0.023 U/ml with 35% reduction in dissolved oxygen) and at 47°C (1.15±0.012 U/ml with 46% reduction in dissolved oxygen) and at 67°C (0.77±0.018 U/ml with 31% reduction in dissolved oxygen) was observed, indicating same trend of activity as of *E. coli*.

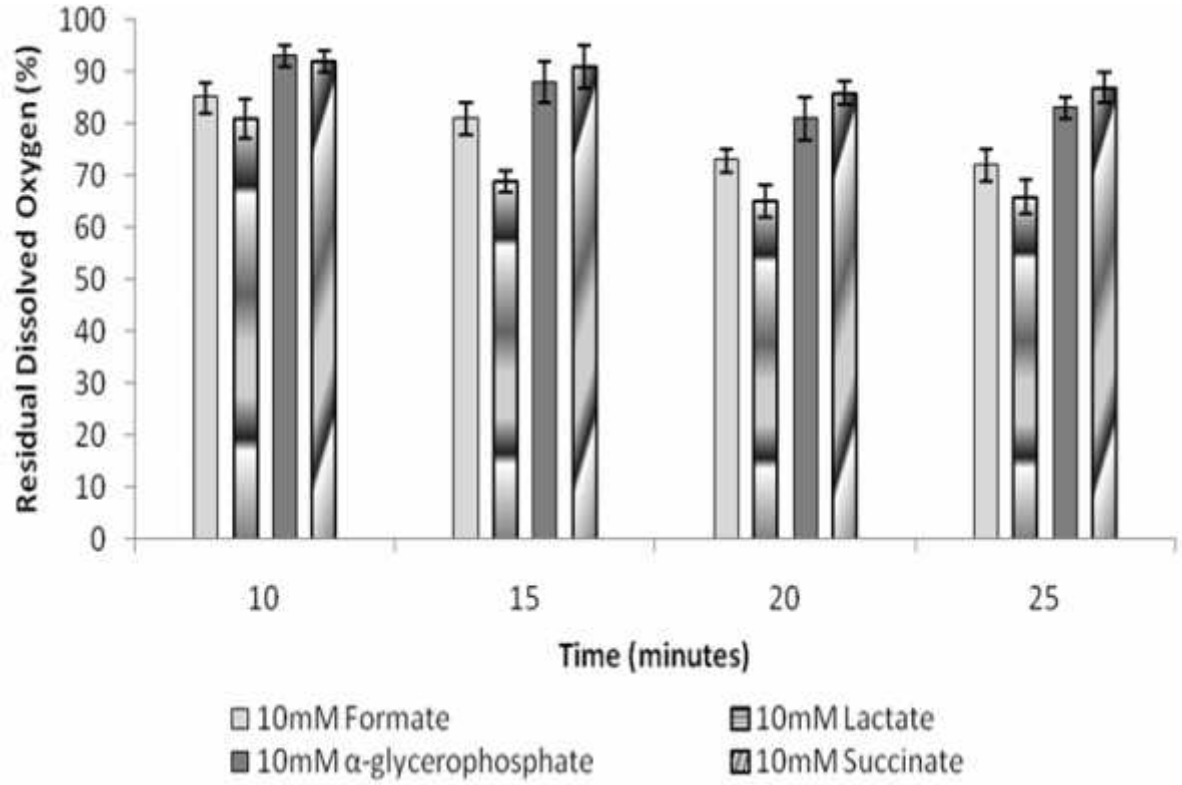

Figure 2. Effect of different substrates/H^+ Donor on oxyrase activity of *Salmonella sp.* (pH 7.5, Temperature 37°C)

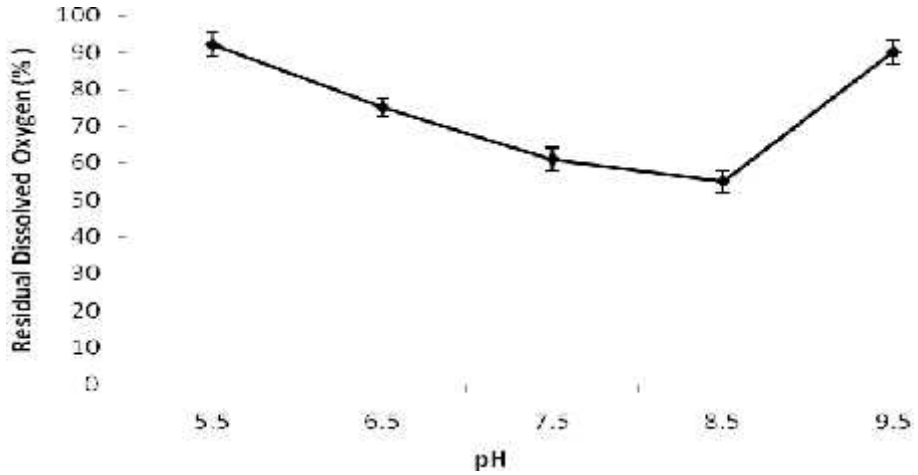

Figure 3. Effect of pH on oxyrase activity of *Escherichia coli* (10mM Lactate as substrate, Temperature 37°C)

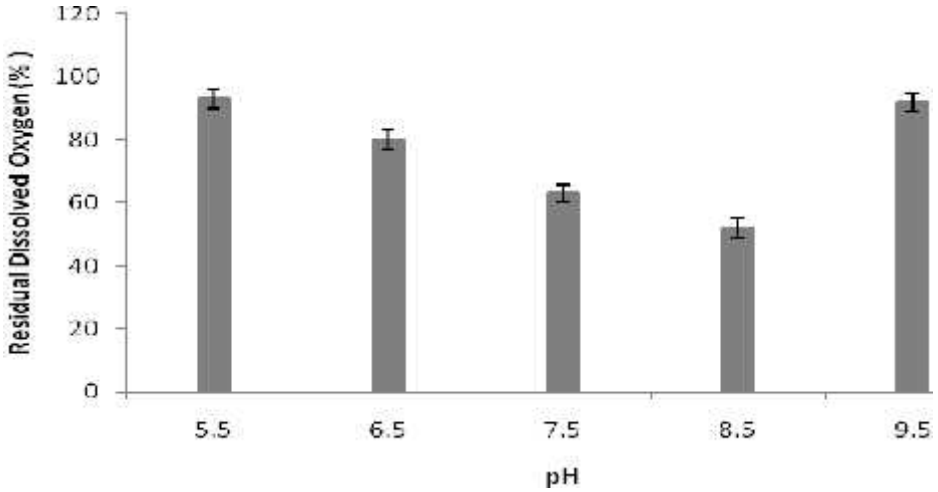

Figure 4. Effect of pH on oxyrase activity of *Salmonella sp.* (10mM Lactate as substrate, Temperature 37°C)

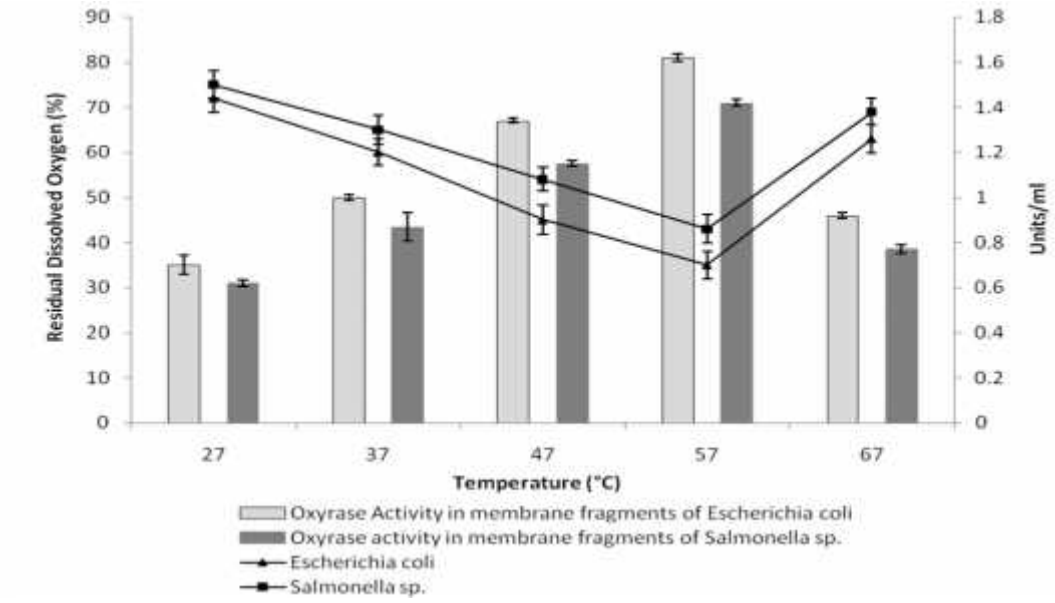

Figure 5. Effect of temperature on oxyrase activity (pH 8.5, 10mM Lactate as substrate)

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