A STUDY OF THE RESPONSES OF SDH, ICD, MDH AND G6PD TO ACID SHOCK GENERATED ACID TOLERANCE IN *Escherichia coli*.

A
DISSERTATION PROJECT
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DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY
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CERTIFICATE

*This is to certify that the thesis entitled "**A Study on the responses of SDH, ICD, MDH & G6PD to acid shock generated acid tolerance in Escherichia coli**" submitted to the Department of Biochemistry & Biotechnology, Institute of Science, in partial fulfillment of the requirement for the award of the Degree of M. Sc. in Biochemistry, is a faithful record of bonafide research work carried out by **Mr. Ashish Kumar Singh** under the guidance of Dr. **Sarika Sinha***

No part of the thesis has been submitted for any other degree or diploma. The candidate possesses minimum 75% attendance in the current academic session.

I further certify that any help or information received during the work on this thesis has been duly acknowledged.

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I dedicate my thesis to all those who have **differed** because:

We owe almost all of our knowledge not to those who have agreed, but to those who have differed.

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Date: _____ Ashish Kumar Singh

ABBREVATIONS

ATP	Adenosine-5-triphosphate
BSA	Bovine serum albumin
Complex I	NADH dehydrogenase
Complex II	Succinate dehydrogenase
Complex III	Cytochrome bc_1 complex
Complex VI	Cytochrome oxidase
Cyt c	Cytochrome c
DCIP	Diclorophenolindophenol
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide (oxidized form)
$FADH_2$	Flavin adenine dinucleotide (reduced form)
G-6-P	Glucose-6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
ICD	Isocitrate dehydrogenase
MDH	Malate dehydrogenase
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
OD	Optical Density
PBS	Phosphate buffer saline
pmf	Proton motive force
PTS	Phosphoenolpyruvate-dependent phosphotransferase system
SDH	Succinate dehydrogenase
TCA	Tricarboxylic citric acid

Contents

ABSTRACT

Only those who will risk going too far can possibly find out how far one can go.

Escherichia coli have an optimum pH range of 6 to7 for growth and survival and are hence, called neutrophiles. When challenged by low pH, protons enter the cytoplasm; as a result mechanisms are required to alleviate the effects of lowered cytoplasmic pH. *Escherichia coli* undergo acid adaptation wherein there is an induction of glutamate, arginine and lysine decarboxylases and RpoS-dependent oxidative systems, which confers acid tolerance. This study examined the activities of SDH, ICD, MDH and G6PD in acid shocked cells of *Escherichia coli* DH5α and *Escherichia coli* W3110 subjected to pH 3, 4, and 5 by different types of acidification, viz. external (using 0.1N HCl), external along with the monensin (1μM) and cytoplasmic (using 20 mM sodium benzoate), for different time periods.

This study highlights new insights into activities of isocitrate, succinate, malate and glucose-6-phosphate dehydrogenases, coupled to the electron transport chain by the reducing power, as yet another system possessed by *Escherichia coli* as an armor against harsh acidic environments. The activities of dehydrogenases in acid shocked cells were monitored at intervals of 1, 2, 3 and 4 h at different pH and then compared with their activities at pH 7 (taken as control).

The results show that an exposure to acidic environment (pH 3, 4 and 5) for a short period of time increased the activities of these dehydrogenases in all types of acidification except cytoplamic acidification used in the current study. In cytoplasmic acidification the activities of all dehydrogenases decreased at pH 3, 4 and 5. On external acidification along with monensin, activities of dehydrogenases increased further as compared to normal external acidification because monensin as an uncoupler destroys the electrical potential of proton motive force. Cells exposed to pH 3 for 2 h had the highest acid tolerance in external acidification with or without monensin. It was also found that activity of glucose-6-phosphate dehydrogenase remained unchanged at low pH. This result suggest that, in a low pH environment, metabolic flux in *E. coli* increases through TCA cycle and remains unaffected through the pentose phosphate pathway. This increase in metabolic flux through TCA cycle, during oxidative phosphorylation, cause electrons from NADH or $FADH_2$ to pass onto O_2 through the electron transport chain located in the plasma membrane of the microorganism, leading to the pumping of protons out of the cytoplasm and thus maintaining pH homeostasis.

Keywords**:** Dehydrogenases, electron transport chain, acid tolerance, external acidification, internal acidification and intracellular pH.

INTRODUCTION

Never tell a young person that anything cannot be done. God may have been waiting for centuries for somebody ignorant enough of the impossible to do that very thing.

In the catabolic metabolism of prokaryotes energy is conserved through either substrate level phosphorylations which are catalyzed in free solution or oxidative phosphorylations which occur within and require the integrity of membranes. The success of the chemiosmotic hypothesis in explaining energy conservation in prokaryotes and in eukaryotes organelles suggests that a gradient of H^+ activity may well be the driving force in all membrane-dependent energy conservation reactions.

The ΔpH across the cytoplasmic membrane is linked to cellular bioenergetics and metabolism of the body which is major supplier of the proton motive force (pmf), therefore homeostasis of cellular pH is essential. According to Mitchell's chemiosmotic hypothesis, the transmembrane electrochemical gradient of protons plays a central role in energy transduction and transport processes in bacteria. These organisms contain membrane-bound proton pumps that generate a transmembrane proton motive force composed of a trans-membrane electrical potential and a transmembrane chemical gradient, so if the influx of proton in the cytoplasm through the membrane bound F_0F_1 ATPase to produce ATP, is left unrestricted, it will rapidly destroy the internal pH away from neutral or near neutral to acidic (MichelsI and Bakker, 1985). As most of the proteins and enzymes are active at near neutral pH, their function is impaired by disturbance in cellular pH. The interference caused by these free intracellular protons impairs processes such as DNA transcription, protein synthesis and enzyme activities (Madshus, 1988).

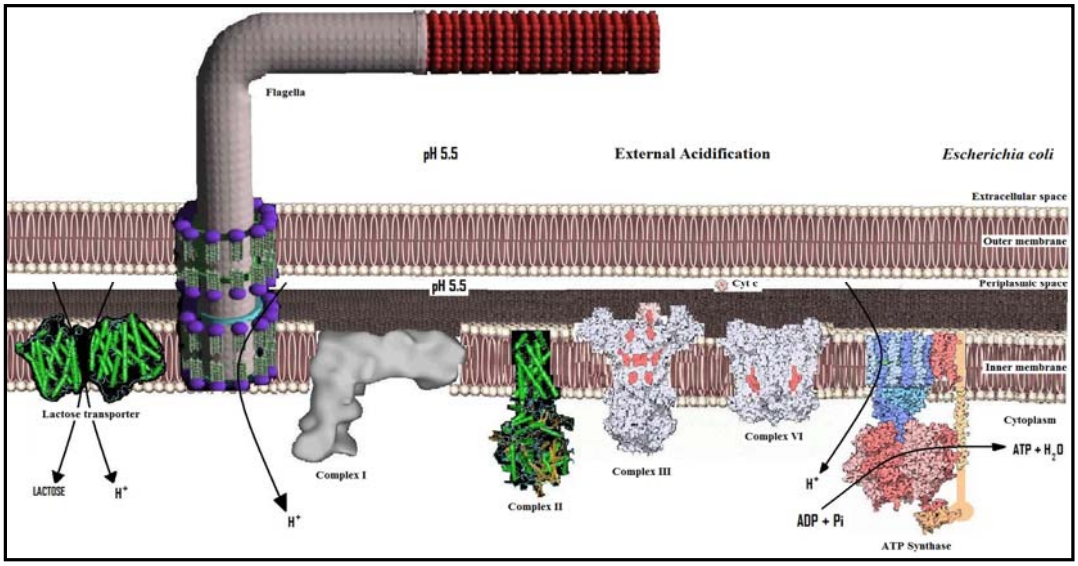

Figure 1: H^+ ions ways of entry into a cell. Few ways through which H^+ ions enter into the cytoplasm on external acidification viz. through lactose transporter, flagellar motion and ATP synthase.

The ability to harness energy and to channel it in to biological work is a fundamental property of all living organisms; it must have been acquired very early in cellular evolution. Carbon fuels are oxidized to yield electrons with high transfer potential. These are NADH and $FADH_2$ which are formed in glycolysis, fatty acid oxidation, and the citric acid cycle which are energy rich molecules because each contains a pair of electrons having a high transfer potential. In oxidative phosphorylation, electrons from NADH or $FADH_2$ are passed onto O_2 through the electron transport chain located in the plasma membrane of microorganism which leads to the pumping of protons out of the cytoplasm (Dawson, A. G., 1979). Then this electromotive force is converted into a proton motive force and, finally, this proton motive force is converted into phosphoryl transfer potential. The conversion of electromotive force into proton motive force is carried out by three electron driven proton pumps (i) NADH-Q oxidoreductase, (ii) Q-cytocrome c oxidoreductase and (iii) cytochrome c oxidase. These large transmembrane complexes contain multiple oxidation-reduction centers, including quinone, flavin, iron-sulphur cluster hemes, and copper ions. Membrane bound enzymes couple electron flow to the production of a transmembrane pH difference, accomplishing osmotic and electric work. The resulting uneven distribution of protons generates a pH gradient and a transmembrane electrical potential that creates a proton motive force. Proton gradients are an interconvertible currency of free energy in biological systems. ATP is synthesized when protons flow back to the cytoplasm through ATP synthase. Thus the oxidation of fuels and the phosporylation of ADP are coupled by a proton gradient across the plasma membrane of microorganisms (Hatefi, Y., 1985).

In biological systems, oxidation is often synonymous with dehydrogenation and many enzymes that catalyze oxidation reactions are dehydrogenases. As a substrate molecule undergoes oxidation (dehydrogenation), giving up two hydrogen atoms, the oxidized form of the nucleotide (NAD^+ or $NADP^+$) accepts a hydride ion ($:H^-$ the equivalent of a proton and two electron) and is transformed into the reduced form (NADH or NADPH). The second proton removed from the substrate is released to the aqueous solvent. The half-reaction for each type of nucleotide is therefore:

$$NAD^+ + 2e^- + 2H^+ \rightarrow NADH + H^+$$

$$NADP^+ + 2e^- + 2H^+ \rightarrow NADPH + H^+$$

Reduction of NAD^+ or $NADP^+$ converts the benzenoid ring of the nicotinamide moiety to the quinonoid form. The reduced nucleotides absorb light at 340nm; the oxidized form does not. The plus sign in the abbreviation NAD^+ and $NADP^+$ does not indicate the net charge on these molecules (they are both negatively charged); rather it indicate that the nicotinamide ring is in its oxidized form, with a positive charge on the nitrogen atom. More than 200 enzymes are known to catalyze reaction in which NAD^+ ($NADP^+$) accept a hydride ion form a reduced substrate, or NADPH (or NADH) donates a hydride ion to an oxidized substrate. These enzymes are commonly called dehydrogenases. Most dehydrogenases that use NAD^+ or $NADP^+$ bind the cofactor in a conserved protein domain called the Rossmann fold. The Rossmann fold typically consists of a six stranded parallel β sheets and four associated α helices.

E. coli W3110 is a negative mutant for phosphotransacetylase (Pta) an important enzyme in the acetate production pathway. The conversion between acetyl-CoA and acetate is mediated by two pathways: (i) acetate kinase (AckA) and phosphotransacetylase (Pta), which rapidly convert acetyl-CoA via acetyl-phosphate to acetate as an overflow pathway and also convert exogenous acetate back to acetyl-CoA, and (ii) acetyl-CoA synthetase (Acs), a high-affinity, low-capacity uptake pathway for acetate which produces acetyl-CoA via an enzyme-bound acetyl-adenylate intermediate (Kirkpatrick *et al*., 2001).

The pta mutation results in a growth defect and the excretion of unusual by-products. Components of the TCA cycle function almost exclusively to provide three precursor metabolites, namely, oxaloacetate (OAA), α-ketoglutarate, and succinyl-CoA. The TCA cycle does not function as an energy-generating cycle. The flux to PEP is insufficient and the insufficiency is resolved by a reverse flux through PEP synthetase, indicating the oversupply of pyruvate. A more likely way of supplying sufficient PEP flux is the uptake of additional glucose and its conversion to acetate (Elwood *et al*., 2008).

However, glucose transportation via PTS and the action of pyruvate kinase generate pyruvate in excess of the required amount for precursor metabolites downstream of pyruvate, namely, acetyl-CoA, α-ketoglutarate, and pyruvate. Oversupplied pyruvate and consequently acetyl-CoA cannot be oxidized completely to CO_2 by the TCA cycle due to the repression of the TCA cycle

enzymes, particularly of α-ketoglutarate dehydrogenase. Therefore, the pyruvate flux should be balanced by opting for acetate excretion. The accumulated acetyl-CoA inhibits the activity of the pyruvate dehydrogenase complex, which causes the accumulation of pyruvate. In response to this metabolic flux perturbation, the *pta* mutant was found to excrete pyruvate, D-lactate, and L-glutamate (Chang *et.al*., 1999).

Sodium benzoate as acid permeant:

Generally used food preservatives are sodium benzoate, propionic acid and sorbitol which retard the microbial growth thereby enhancing the shelf life of food. Sodium benzoate acts as a preservative of food and in packed food products by causing cytoplasmic acidification of bacterial cells at low pH. Addition of a permeant acid such as benzoate at high concentration depresses cytoplasmic pH with little or no recovery, without affecting external pH. On addition of sodium benzoate as an acid permeant to cell cultures which were suspended in media at pH adjusted to acidic range, the cytoplasmic pH fell within seconds to that of the external medium (Kanna *et al*., 2008).

Monensin as an uncoupler:

According to the chemiosmotic theory of Mitchell, bacteria use membrane-bound ATPases or electron transport system to translocate protons across the cell membrane. Proton extrusion establishes a proton motive force (pmf) which is a summation of chemical (ΔpH) and electrical (ΔΨ) potential gradients. Ionophores, like monensin are highly lipophilic substances which are toxic to many bacteria, protozoa, fungi, and higher organisms. The exterior of the molecule is hydrophobic, while the interior is hydrophilic and able to bind cations. Some ionophores bind only a single cation (uniporters), but others are able to bind more than one cation (antiporters). Because cell membranes are composed of lipid bilayers, high activation energy is needed to translocate ions. Ionophores function as mobile carriers within the membrane and able to shield and delocalize the charge of ions thereby facilitating their movement across membranes. Monensin is an antiporter with a high selectivity for Na^+ than K^+ (Pressman, B. C. 1976).

The hypothesis behind the dissertation work:

Most of the bacteria survive at neutrophilic pH, but when stressed to acidic condition many machinery of acid tolerance response present enable them to survive the harsh acidic environment. But still there are many such mechanisms that are unexplored and need to be investigated. Our dissertation work on acid tolerance mechanism of *E. coli* is based on the hypothesis that a higher recycling of cell's reducing power takes place between the central metabolic pathway and the ETC in low pH environment. Such a recycling of reducing power results in pumping out of protons from the cytoplasm through the electron transport chain complexes, thereby restoring the cytoplasmic pH of the bacteria in the range of 7.4 to 7.8.

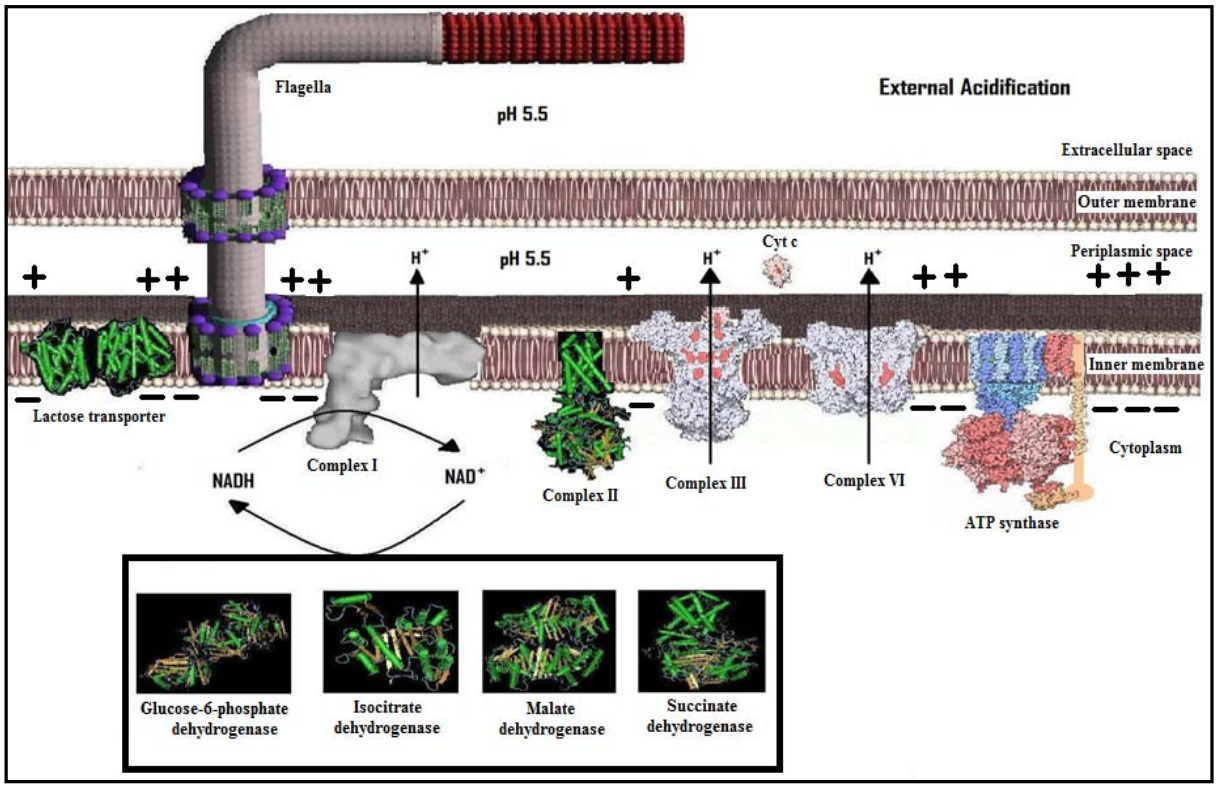

Figure 2: Proposed hypothesis in maintenance of pH homeostasis. On external acidification of *E. coli*, H^+ ions enters into the cytoplasm rapidly which are pumped out through the ETC by the continuous recycling of reducing power between TCA cycle dehydrogenase enzymes and the ETC.

Our dissertation work was conducted in three parts:

PART 1: Investigating the activities of SDH, ICD, MDH and G6PD in *Escherichia coli* DH5α in acid shock conditions of pH 3, 4, and 5 for time periods of 1, 2, 3 and 4 h.

i. External acidification by using the 0.1N HCl.
ii. External acidification along with 1μM monensin (uncoupler).

PART 2: Investigating the activities of SDH, ICD, MDH and G6PD in *Escherichia coli* W3110 in acid shock conditions of pH 3, 4, and 5 for time periods of 1, 2, 3 and 4 h.

i. External acidification by using the 0.1N HCL.
ii. External acidification along with 1μM monensin (uncoupler).

PART 3: Investigating the activities of SDH, ICD, MDH and G6PD in *Escherichia coli* DH5α and *Escherichia coli* W3110 in acid shock conditions of pH 3, 4, and 5 using sodium benzoate for time periods of 1, 2, 3 and 4 h.

REVIEW OF LITERATURE

Some people spend their entire lives reading without ever being able to go any further. They don't understand that words are just stones across a river. The words are there only to reach the other side. It is the other side that matters.

Most of the microorganisms are neutrophiles because they survive only within the range of 5 to 8.5 pH and exhibit maximum growth rate at neutral or near neutral pH. The ability of neutrophilic bacteria to survive under acidic condition is essential for successful colonization in the mammalian host because low pH environment leads to cytoplasmic acidification in microorganism. Therefore, acidic condition causes growth arrest and bacterial death due to the changes in macromolecular structure; disruption of enzymatic reactions and membrane potential (Jeong *et al*., 2008). To avoid such deleterious effects of lowered extracellular pH bacteria have developed several mechanisms, which are:

1. A cellular envelope modification to make smaller ionic permeability or decrease in membrane fluidity (Benjamin & Datta, 1995; Dilworth & Glenn, 1999).
2. Induction of DNA repair machinery and chaperones protein which result in major change in gene expression and overexpression of some gene (Bearson *et al*., 1997).
3. The development of ionic pumping systems and proton extrusion/uptake systems.
4. Increasing the external pH indirectly, which involves the decarboxylase activities of glutamate, arginine and lysine (Hommais *et al*., 2004).

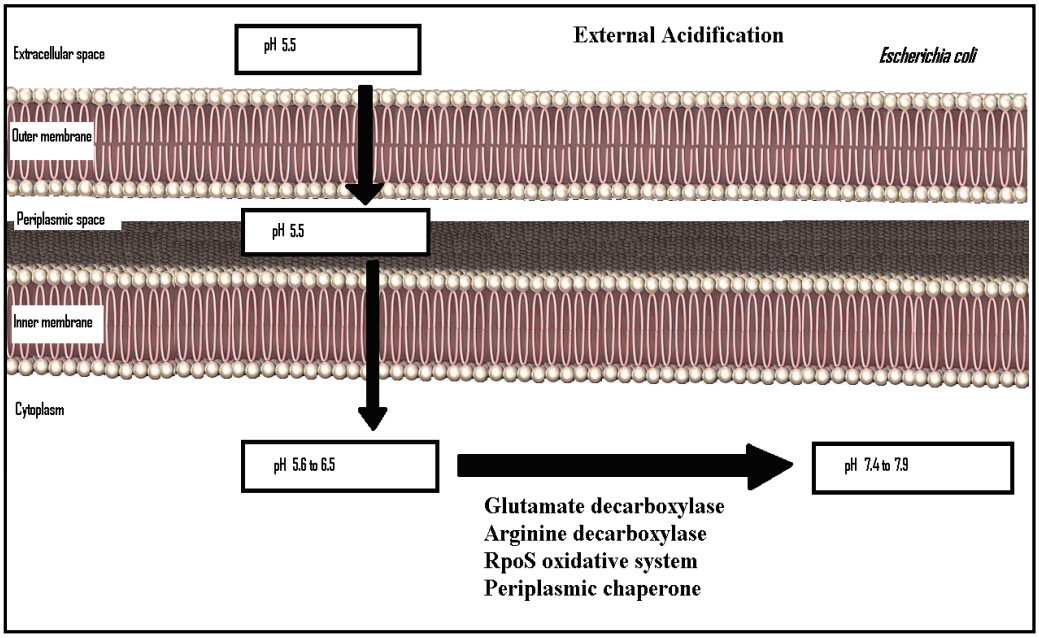

Figure 3: **Mechanisms of maintaining pH homeostasis.** Few worked out mechanisms of acid tolerance in neutrophiles which includes amino acid decarboxylase, DNA repair protein and RpoS oxidative system and are involved in pH homeostasis during acidic condition.

When the external pH of *Escherichia coli* cells in suspension was lowered from pH 7.5 to 5.5, the cytoplasmic pH fell within 10 to 20 s to pH 5.6 to 6.5. Rapid recovery occurred after 30 s of HCl addition and was followed by a slower recovery over the next 5 min. The pH of the periplasm equaled the external pH under all condition tested, including rapid acid shift. Benzoate addition to the cell suspension had no effect on the periplasmic pH (Wilks and Slonczewski, 2007). Uptake of a permeant acid can also dissipate the transmembrane pH, a component of the proton motive force (pmf). The permeant acids, such as sodium benzoate, can cross the bacterial membrane in the protonated and unprotonated forms and cyclically run down ΔpH, thereby collapsing pmf and uncoupling ATP synthesis (Lambert *et al*., 1997).

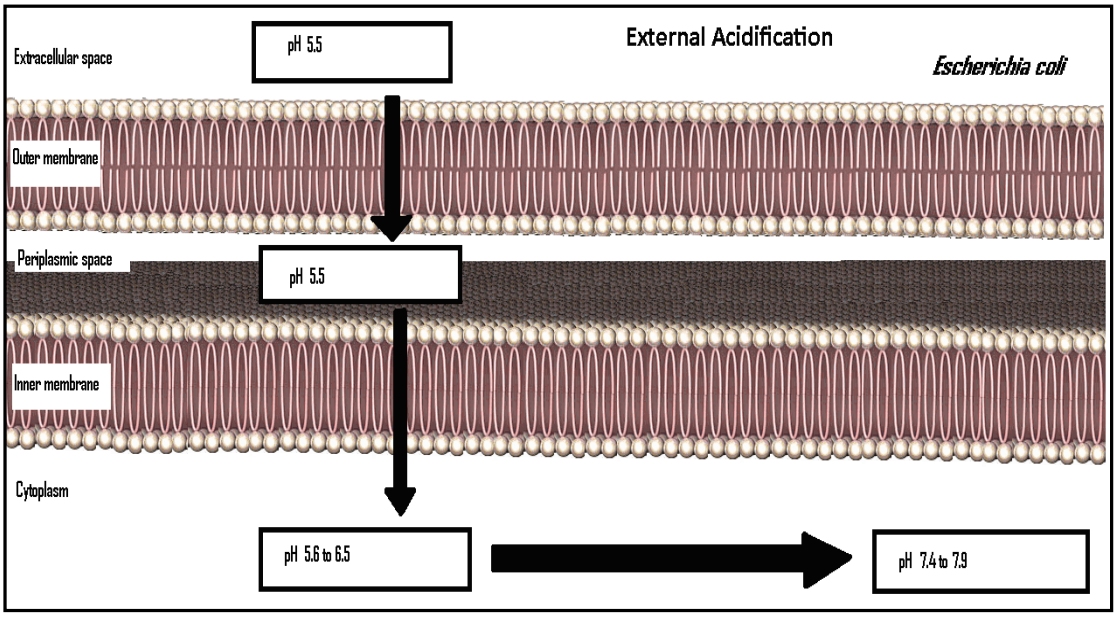

Figure 4: Periplasmic pH of *E. coli* on external acidification. *E. coli* periplasmic pH equals external pH under types of external acidification and that it is not affected by sodium benzoate.

Coordinated regulation of genes expression in response to environmental stimuli is an important requirement for adaptation of bacteria to the various environments. One of the major mechanisms of signal transduction leading to specific genes expression in bacteria is the two-component system, which consist of a sensor kinase and its cognate response regulator (Masunda *et al*., 2003). The main defense strategies protecting the cell from acid are changes in membrane composition, homeostasis system of internal pH and pathways to repair or protect the essential cellular component.

Under mild acid shock condition bacterium change the cell surface hydrophobicity and also induces a change in the composition of outer membrane protein to maintain pH homeostasis (Dilworth and Glenn, 1999). In most of the bacteria low pH condition induce mechanisms that are involved in the synthesis of degradative amino acid decarboxylases (Dilworth and Glenn, 1999). There are four levels of acid tolerance which are log phase cells, acid adapted log phase cells, stationary phase cells and acid adapted stationary phase cells. During acid shock specific proteins encoded by specific genes are called acid shock proteins. These proteins are required for acid adaptation of log and stationary phase cells (Foster, 2001).

Acid induced decarboxylase activity:

These systems provide acid resistance by consuming intracellular protons via the amino acid decarboxylation reaction and also by gradual alkalinization of the medium. The decarboxylases consume an intracellular proton while removing CO_2 from their amino acid substrates. Decarboxylation products are then expelled by specific antiporters in exchange for new substrate.

These decarboxylase activities include the activities from glutamate, arginine and lysine decarboxylases, which enhance the growth under acid condition by neutralising the medium. (Dilworth & Glenn, 1999; Small & Waterman, 1998) The decarboxylation products from these decarboxylases are μ-amino butyric acid (GABA), agmatine and cadaverine, respectively, which are expelled from the cell by system-specific anti-porters (GadC for GABA, AdiC for agmatine and CadB for cadaverine) in exchange for new substrates (Gong *et al*., 2003; Iyer *et al*., 2003). Thus, these systems protect the cell from acid stress by consuming intracellular protons during each decarboxylation reaction. The removal of intracellular protons is used to enhance pH homeostasis and allow the cell to maintain an internal pH of neutral to near neutral.

Acid induced Glutamate dependant decarboxylase activity:

This acid induced decarboxylase system consists of three genes, i.e., *gadA*, *gadB* encoding highly homologous glutamate decarboxylase isoform the gene *gadC* encoding a putative glutamate: GABA antiporter. These two genes *gadA* and *gadB* are 2,100 kbp, distantly located and differing by only 5 amino acid residues (De-Biase *et al*., 1996). The two isoforms of

glutamate decarboxylase - GadA and GadB convert intracellular glutamate to μ-amino butyric acid thereby consuming a proton, simultaneously GadC exports GABA, the end product out of the cell along with the simultaneous import of more glutamate into the cell as substrate. These conversion changes internal pH and electrical charge in ways that enable the cell to survive extreme acid stress conditions so far by maintaining the pH homeostasis (Richard and Foster, 2004).The transcriptional control of gad expression is controlled by an operon and on a number of factors which differ depending upon the growth phase and environment which includes H-NS, RpoS and CRP-cAMP (Castanie and Foster, 2001; De-Biase *et al*., 1999; Rowbury, 1997; Rowland *et al*., 1984).

GadE is a central regulator which is involved in the regulation of several genes required in the regulation of pH homeostasis. GadE has a potential helix–turn–helix DNA-binding motif domain between amino acid 131 and 152 that regulates target genes by binding to the promoter region of these target *gadA* and *gadB* genes (Hommais *et al*., 2004).

Overexpression of *gadX* and *gadE* gene significantly induces the expression of genes that are involved in:

1. Bacterial acid response (*gadA*, *gad E*).
2. Biosynthesis of bacterial membrane protein and lipids.
3. Biosynthesis of glutamate (*gltD*, g*lnH*).
4. Other stress responses gene or gene encoding for chaperone protein (*hdeA*, *hdeB* and *rfaG*) (Hommais *et al*., 2004).

gadX gene activates the expression of *trmE* because of its role in tRNA modification, encodes an Era-like GTPase that is required for *gadA/BC* expression and also activates *gadE* gene (Gong and Foster, 2004). The histone-like protein H-NS influences a variety of cellular processes, such as replication, transcription and recombination, and is a major component of the bacterial nucleoid. This nucleoid protein H-NS is involved in the negative control (Yoshida *et al*., 1993). The mechanism underlying gene regulation by H-NS is due to either change in DNA supercoiling or to transcriptional silencing. The transcriptional silencing occurs through

preferential binding to AT-rich curved DNA sequences which found upstream of *E. coli* promoters (Waterman1 and Small, 2003).

Change in membrane lipid composition:

In most of the bacteria low pH condition induces change in the composition of lipids and the decrease in membrane fluidity which may increase acid resistance. Membrane fluidity is important for cells because it affects membrane functions such as biochemical reactions, transport systems, and protein secretion. The decrease in membrane fluidity under acidic growth conditions may be associated with changes in proton flux so that acid adapted cells do not allow protons to flow into the cell as easily as non adapted cells (Yuk and Marshall, 2004). The fatty acids which are most influenced by the pH in most of the bacteria are palmitic (16:0), *cis*-vaccenic (18:1ω7c), cyclohepta-decanoic (C17:0), and cyclononadecanoic (C19:0) acids. Acid adaptation increases the amounts of palmitic acid (C16:0) and decreases *cis*-vaccenic acid (C18:1ω7c) in the lipid membrane of most the bacteria during acidic condition (Brown *et al*., 1997).

Acid adaptation or inducible acid tolerance means microorganisms show an increase resistance to acidic environment after exposure to acidic condition. The gradual increase in acidic condition allow microorganisms to induced acid resistance (habituation to acid) allowing habituated organisms to stay alive even survive subsequent exposures to acidic conditions which are hazardous to non-habituated cells (Goodson and Rowbury, 1989). Habituation in most of the bacteria involves a phenotypic change in organisms which gain acid resistance after an exposure to low pH for a short time. Such development of acid resistance is dependent relative on protein synthesizing machinery and number of proteins that are induced at low pH (Raja *et al*., 1991). The cells which are acid shock increase the acid tolerance of cell at acidic pH, however the cells which have undergone acid adaptation, acid tolerance is not induced in those cells at acidic pH. In acid shock cells the bacteria exposed to acid at pH 4.5 for 2 hour had highest acid tolerance (Tosun and Gonul, 2005).

DNA damage is prevented by Dps binding:

The most important protein that contributes to acid tolerance of *E. coli* is the DNA binding protein in starved cell (Dps). Dps protected DNA from acid damage by binding to DNA. Low pH appeared to strengthen the Dps-DNA association and the secondary structure of Dps retained or formed α-helices at low pH. The mechanism by which Dps protein protect the DNA is formation of spherical dodecamers complex, similar to that of ferritins complex. This DNA-Dps stable complex protects the DNA from the hydroxyl radicals formed during oxidative stress (Choi *et al*., 2000). Dps protein protects the DNA from acid damage by binding to DNA and at low pH condition appears to build up the Dps-DNA association and restored the DNA-binding activity of heat-denatured Dps. The secondary structure of Dps protein retains or forms alpha helices at low pH condition (Jeong *et al*., 2008). Dps protein also plays an important role in survival during other stress conditions including starvation, near UV and gamma irradiation, thermal stress, metal toxicity and oxidative stress (Jeong *et al*., 2008). In some cases highly ordered nucleoprotein complexes are capable of altering gene expression pattern when Dps binds to DNA in acidic condition. Acid challenge of whole cell results in chromosomal DNA damage and this damage increases with increase in exposure time. Dps also influences expression of other genes that protect or repair DNA or provide acid tolerance. Dps is a key component of the general stress protection system that is important in the survival of the bacterium in acidic condition (Choi *et al*., 2000).

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DNA repair pathway:

In bacterial cell the low pH condition damages chromosomal DNA as a result of selective depurination or by breakage in double stranded DNA. As a result of such depurination creation of unrepaired DNA and mismatch sequences leads to the death of cells and therefore DNA repair pathways are important for cell survival in low-pH conditions (Raja *et al.,* 1991). DNA repair pathway includes repair mechanism such as *recA* and *uvrB* which also help in survival during extreme gastric acidity. These DNA repair process are responsible may be the same or interconnected to different factor that leads to increased UV-resistance and increased repair of UV-damaged DNA in acid-habituated neutrophilic bacteria (Goodson and Rowbury, 1989).

Inducible acid tolerance system:

A sub-lethal environmental acid stress (pH 4.5 to 5.5) provides protection against subsequent exposure to a lethal stress (pH < 4.0) by inducing an adaptive tolerance response in many bacteria known as acid tolerance response (ATR) (Dependiene *et al*., 2005).

Acetate induced acid tolerance:

Acetate is one of the major by-products of *Escherichia coli* metabolism. The global gene expression changes seen after adaptation to external acetate, suggests regulation of carbon metabolism in *Escherichia coli* to avoid further formation of acetate. Long-term adaptation to acetate resulted in a decreased expression of gene belonging to operon coding for protein involved in the uptake and initial degradation of carbon sources other than glucose, e.g., maltose, trehalose, galactose, melibiose, rhabinose, fucose, ribose, arabinose, the sugar alcohol glycerol, the hexuronates, and the amino acid sugar N-acetyl glucosamine and N-acetylneuraminate. In addition to these, genes coding for the uptake of lactate (*lldP*) were decreased in the presence of acetate (Pollen *et al*., 2003).

Insufficient carbon flux through the citric acid cycle due to repression of this cycle in rapidly growing cells has been proposed to prompt acetate production (Lee *et al*., 1994). Metabolic engineering to reduce acetate formation mainly resulted in the redirection of metabolism towards other by-product (Aristidon *et al*., 1995; Farmer and Liao, 1997; Chang *et al*., 1999). Acetate is transported into the cells and activated to acetyl-CoA by acetyl-CoA synthetase (encoded by *acs*). The expression of this enzyme increases in presence of acetate and is regulated by cyclic AMP (cAMP), receptor protein and FNR as well as its activator, FadR (Polen *et al*., 2003). As pH falls, *E. coli* limits internal acidification from metabolism, by producing lactate instead of acetate plus formate, by reuptake and activation of acetate to acetyl-CoA which enters into the tricarboxylic acid (TCA) cycle, and by conversion of formate to H_2 and CO_2 (Kirkpatrick et al., 2001).

The other gene, whose expression is induced by exposure to acetate at neutral or near neutral pH, codes for a protein that protects the cells against oxidative damage caused by superoxides and hydrogen peroxide. The *cfa* gene which is acetate induced codes for a cyclopropane fatty acid

synthase, which is involved in the modification of the phospholipids profile by adding a methylene group across the carbon-carbon double bond of unsaturated fatty acids in the inner membrane. The product of another acetate – induced gene is *grxB* gene. This *grxB* gene codes for glutaredoxin 2, which is glutathione-dependant oxidoreductase (Arnold *et al*., 2001). The increased tolerance of stationary-phase cells to hydrogen peroxide challenge is due to the production of catalase. Bacteria possess three separate catalase genes (*katG*, *katE*, and *katP*). KatG is regulated by OxyR and mostly produced during log phase, while *katE* is primarily expressed in the stationary phase and regulated by RpoS which code for catalase hydroperoxidase (Choi *et al*., 2000). After addition of acetate there are many other genes whose relative expression level is decreased by at least 50% at the transcription-translation machinery. The majority of these genes encode a total of 41 ribosomal proteins, as well as the translation elongation factors EF-G, EF-Ts, and EF-Tu (Arnold *et al*., 2001).

Role of RpoS in acetate induced acid tolerance:

In most of the neutrophiles the RpoS gene, which codes for the alternate sigma factor σ, is required for both stationary and exponential phase induced acid resistance when neutrophiles grow at moderately low pH (Arnold *et al*., 2001). Among the genes, whose expression is strongly induced by acetate, is *yhiW*, which codes for Ara-c type regulating protein. This RpoS transcription factor may be involved in controlling some of the changes in gene expression in acidic condition. The *rpoS* gene regulates *cfa* transcription, which activates CFA synthase and so *rpoS* has been termed as a global stress regulon (Tosun and Gonul, 2005). The genes whose expression is changed after acetate adaptation is RpoS dependent general stress response genes (*osmY*, *otsAB*, *poxB*, *dps*, and *hdeAB*) (Arnold *et al*., 2001).

The reducing equivalent of the cell:

The main reducing equivalent synthesized by the cell central metabolism is NADH, making it the principal electron donor to respiratory chains. Moreover, the [NADH]/[NAD^+] ratio is responsible for the regulation of some cell pathways leading to the synthesis of ATP (Nelson and Cox, 2005). The cell requirement of NAD^+ makes NADH turnover a top priority over ATP synthesis (Berg *et al*., 2002). In prokaryotes, NADH is produced in the cytosol, mainly by the glycolytic enzymes glyceraldehydes-3-phosphate dehydrogenase, pyruvate dehydrogenase, and

three enzymes in the tricarboxylic acids cycle: isocitrate, α-ketoglutarate, and malate dehydrogenases (Berg *et al*., 2002). In addition to its prominent role in energy production, NADH is a potential source of NAD^+, the main cellular oxidant. Since the amount of NAD^+ is small in comparison with the amount of substrates being oxidised, the NADH resulting from the oxidative reaction must be rapidly reoxidised, restoring the NAD^+ level, to ensure that the oxidation is not limited by the lack of NAD^+ (Dawson, 1979). The NADH:quinone oxidoreductase, also called rotenone sensitive NADH dehydrogenase (complex I) (NDH-1), is the largest complex of the respiratory chain. Along with NDH-1, Type II NADH:oxidoreductase, also called rotenone-insensitive NADH dehydrogenase (NDH-2) is present in the electron transport chain of *Escherichia coli* (Bjorklof *et al.,* 2000) with the absence of Type III NADH dehydrogenase (Nqr), the so-called Na^+-translocating NADH:quinone oxidoreductases.

Rapid acidification:

Many of the Escherichia coli genes in acidic (pH 5-6) or in the basic (pH 8-9) show pH dependent expression during logarithmic growth. Rapid acid treatment could distinguish between genes responding to external pH, and genes responding to cytoplasmic acidification, which occur transiently following rapid external acidification. When cultures of *Escherichia coli* are subjected to rapid acid treatment, the cytoplasmic pH decreases but begins to recover within less than a minute after the acid shift.

On acid treatment 630 genes were up-regulated and 586 genes were down-regulated. Up regulated genes included amino acid decarboxylases, succinate dehydrogenase, biofilm-associated genes and gad, fur and rcs regulons. Genes responding to cytoplasmic acidification were biofilm developmental genes *ymgABC* and flagellar phase variation modulator FinB. The delayed up-regulated genes responding to rapid acid treatment were the *nuo* operon encoding NADH dehydrogenase I and the *hsl* genes (Kanna *et al*., 2008).

Mechanism of action of sodium benzoate:

Sodium benzoate is the sodium salt of benzoic acid. It is a white, crystalline solid and is a hygroscopic material. Therefore, the container must be kept closed when not in use. It will easily dissolve in water, forming a transparent, colorless solution. Generally, sodium benzoate is listed

as GRAS (generally recognized as safe) by the U.S. FDA when used as an antimicrobial agent or as a flavoring agent and adjunct in levels not to exceed good manufacturing practice (GMP), currently limited to 0.1% maximum in food. The effectiveness of sodium benzoate as food preservative increases with decreasing pH (increasing acidity). This is because the ratio of undissociated (i.e., free) benzoic acid to ionized benzoic acid increases as the pH decreases. It is generally accepted that the undissociated benzoic acid is the active antimicrobial agent. Because it only converts to benzoic acid in acidic environments, it is not used for its anti-microbial action unless the pH is below about 3.6. Sodium benzoate is not recommended as a preservative at pH

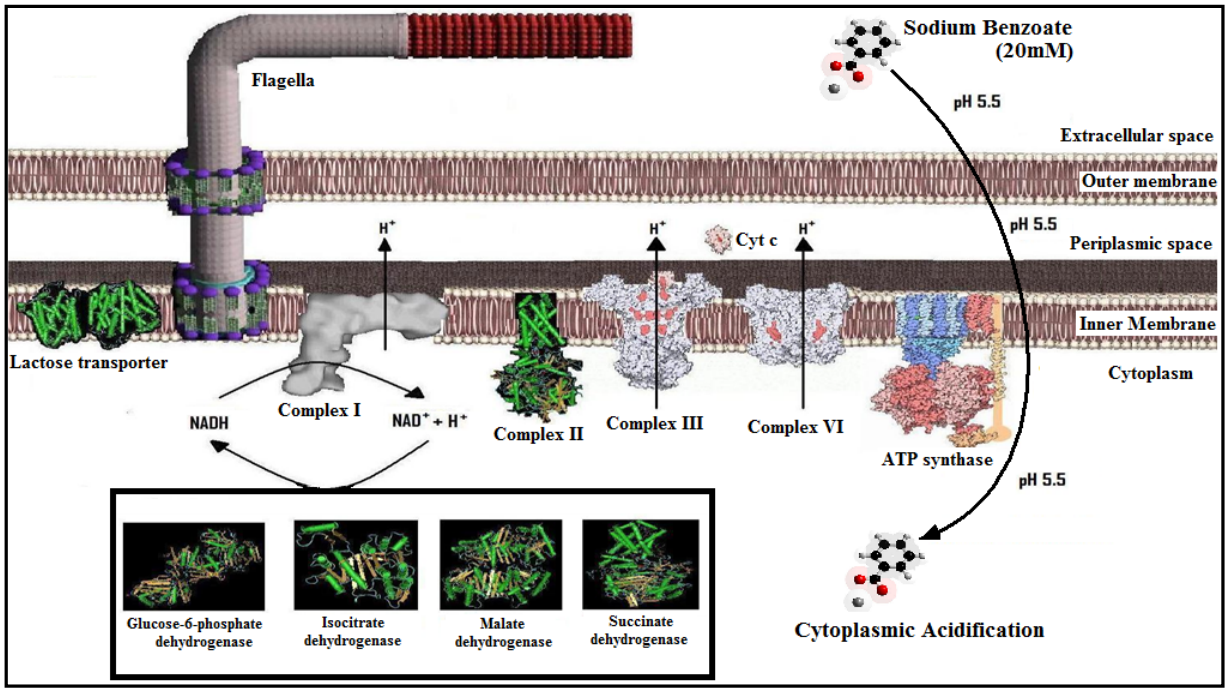


Figure 5: Cytoplasmic acidification using sodium benzoate. In cytoplasmic acidification, sodium benzoate acts as an acid permeant and thereby acidifies the cytoplasm under low external acidic condition. On internal acidification cells pump out the extra H^+ ions through the electron transport chain.

ranges higher than 4.5. The strong dependence of uptake on pH was because of the relative distribution of undissociated and dissociated forms in the solution, not on the pH itself. It is the undissociated part of the acid that more readily enters the cell. The undissociated benzoic acid molecule can move across the membrane where they donate a proton thereby resulting in a net accumulation of protons inside the cytoplasm, which declines the intracellular pH and collapsing the pmf. The cell uses many mechanisms to nullify this effect, one of them is - its membrane

bound ATPase which re-establishes the gradient, depleting the ATP, consequently cell gets de-energized and stops growing.

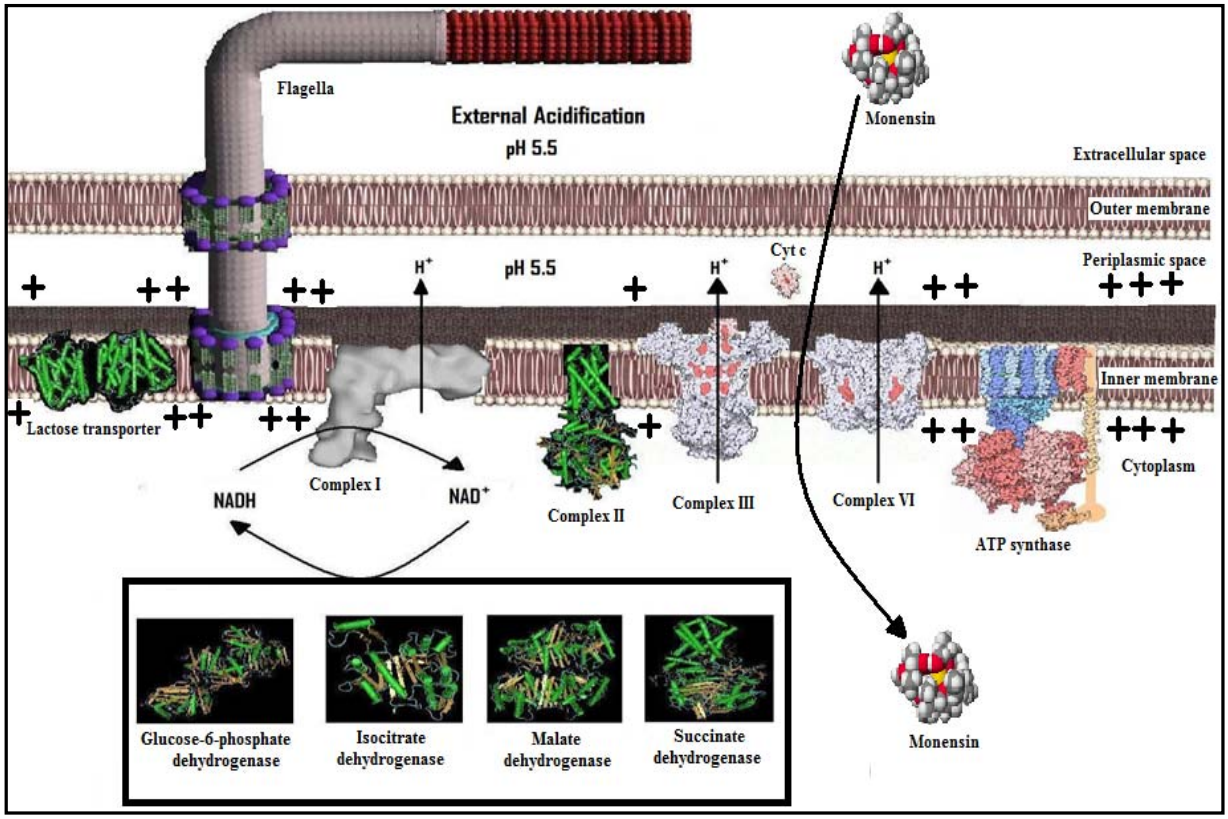


Figure 6: The act of uncoupling of oxidative phosphorylation by monensin. The proton motive force, generated across the inner membrane of *E. coli*, is a summation of electrical and chemical potentials [pmf = $\Delta\Psi + \Delta pH$]. Monensin binds with Na^+ ions in the extracellular space and shuttles them into the cytoplasm of the bacterium. In this way it disrupts the electrical potential ($\Delta\Psi$) and thus, acts as an uncoupler of oxidative phosphorylation.

Monensin (an uncoupler):

In 1967, a group led by Amelia Agtarap isolated monensic acid, now known as monensin, from *Streptomyces cinnamonensis*, and elucidated its intricate structure. Monensin is not only of immense agricultural significance, but also of great chemical importance due to its intricate and oxygen rich structure. Ionophores, a large and structurally diverse set of compounds, have the ability to complex cations (positively charged ions) and assist in their translocation through lipophilic environment that are normally hostile to these charged species.

Coccidial parasites are single-celled protozoa that infect the intestines of young farm animals, causing severe damage to the epithelial lining of the gut and leading to diarrhea, poor nutrient absorption, imbalances of both iron and water. These factors culminate in poor growth and low meat quality in the affected animals. Monensin has a broad-spectrum anticoccidial activity and plays a second important role in improving digestion and, therefore, growth potential in ruminant animals, especially cattle. Monensin, marketed under the brand name Rumensin®, has been used for the last thirty-five years all over the world as a food additive to prevent coccidial infection in susceptible farm animals. The ionophore antibiotics work by disrupting the movement of ions across the bacterial membrane. The bacteria must then work harder, expending a great deal of additional energy, in order to maintain intracellular pH and ion balance. Of the twenty-six carbon atoms which comprises the backbone of this molecule seventeen are asymmetrical substituted, including one specially tough section where six such carbon are arranged contiguously. In both, crystalline and in solution form, it has a cyclic structure maintained by two intramolecular hydrogen bonds between the terminal carboxylic acid residue at one end and the two hydroxyl groups at the other. This organization produces an arrangement whereby the external surface of the molecule is a largely hydrocarbon, lipophilic region, while the many Lewis basic functional groups (all oxygen based) are directed towards the interior. This provides an ideal environment for the coordination of metal cation, making monensin a perfect ionophore (Agtarap *et al*., 2008).

MATERIALS AND METHODS

It is not the hours you put in your work that count, it's the work you put in the hours.

The greatest pleasure in life is doing what people say you cannot do.

1. Microbial strains:

Pure cultures of different *E. coli* strains were obtained from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India, viz.

1. **MTCC1652 - *Escherichia coli* DH5α**
2. **MTCC50 - *Escherichia coli* W3110**

2. Method for revival of cultures:

1. All the cultures were obtained as lyophilized cultures from IMTECH, Chandigarh.
2. Cultures were activated by incubating at 37˚C for 10 min.
3. All cultures were mixed with 300 µL broth and100 µL culture was inoculated in 250 mL broth and then cultures were streaked on nutrient agar plates and slants.

2.2*Escherichia coli* culturing media: *Escherichia coli* cells were grown in M9 minimal media.

3.1 Materials required:

3.1.1 M9 minimal media composition (per liter):

Use: For the cultivation and maintenance of *Escherichia coli.*

pH 7.0 ± 0.2 at 25^{o}C

Na_2HPO_4	6.0
KH_2PO_4	3.0
NaCl	1.0
NH_4Cl	0.5
Carbon source	10.0 mL
$MgSO_4.7H_2O$	1.0 mL
$CaCl_2.2H_2O$	1.0 mL
Thiamine-HCl	1.0 mL

3.1.2 Nutrient media:

pH 7.4 ± 0.2 at 25°C

Source: This medium is available as a premixed powder from Hi Media.

Beef extract	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1.0 g

3.1.3 Luria barteni broth:

pH 7.0 ± 0.2 at 25°C

Pancreatic digest of casein	10.0 g
NaCl	5.0 g
Yeast extract	5.0 g

3. Biochemical test for *Escherchia coli*:

4.1. Gram's staining:

4.1.1. Principle:

Gram staining is based on the physiochemical nature of cell wall of bacteria. Cell wall of gram-negative bacteria is generally thinner than those of gram-positive bacteria. Gram–negative bacteria possess higher percentage of lipids in their cell wall as compared to gram- positive bacteria.

During staining the primary stain crystal violet forms complex with mordant iodine (CV– I) in the cell wall. When gram-positive bacteria are decolorized with ethanol,

the alcohol is thought to shrink the pores of the thick peptidoglycan. Thus, the dye - iodine complex is retained during the short decolorization step and the bacteria remain violet. In contrast, gram-negative peptidoglycan is very thin, not as highly cross-linked and has larger pores. Alcohol treatment also may extract enough lipids from the gram-negative wall to increase porosity further. For these reasons, alcohol more readily removes the crystal violet-iodine complex from gram-negative bacteria. These cells subsequently take color of counter stain - the safranin.

4.1.2 Reagents required:

1. Crystal violet
2. Iodine solution
3. Gram stain decoloriser (95% ethanol)
4. Safranin

4.1.3 Procedure:

1 or 2 loopful of broth were applied to a clean slide. It was then air dried and later heat fixed. Later stained with crystal violet for 1 minute and was rinsed with water. Next iodine solution was applied for 1 minute and was rinsed with water. After that it was carefully decolorized with 95% ethanol. Then immediately, was rinsed with water. Further counterstained with safranin for 30 second and finally was again rinsed with water. Observation under 100X oil emersion lens and pink colour cells were observed concluding its gram nature.

4.2 The Indole Test:

4.2.1 Principle:

The test organism is inoculated into tryptone broth, a rich source of the amino acid tryptophan. Indole positive bacteria such as *Escherichia coli* produce tryptophanase, an enzyme that cleaves tryptophan, producing indole and other products. When Kovac's reagent (p-dimethylaminobenzaldehyde) is added to a broth with indole in it, a dark pink color develops.

4.2.2 Reagents required:

1. Kovacs reagent
2. Peptone water

4.2.3 Procedure:

One loop of culture in brilliant green bile broth (BGBB) was transferred to a tube of peptone water. The tubes were then incubated at 44° C ± 0.5 in a water bath for 24 to 48 hours. After incubation of the inoculated tubes, the presence of indole was shown by a red color in the alcohol phase of the Kovacs reagent, added at 0.5 mL per tube.

5. Acid shock method:

E. coli DH5α and *E. coli* W3110 were given acid shock at different pH ranges for different time intervals.

5.1. External acidification:

1. Single cell colony of both bacteria was inoculated in 250 mL M9 minimal media adjusted to pH 7 and was incubated at 37^{o}C.
2. When the optical density (OD) of the inoculated cultured broth reached A_{600nm} = 0.8 to 1.1, cells were collected by centrifuging at 10,000 rpm for 5 minute.
3. The collected cells were then subjected to acid shock in M9 minimal media with different pH of 3, 4 and 5 for incubation time periods of 1h, 2h, 3h and 4h.

5.2. External acidification along with monensin:

1. Single cell colony of both bacteria was inoculated in 250 mL M9 minimal media adjusted to pH 7 and was incubated at 37^{o}C.
2. When the optical density (OD) of inoculated culture media reached A_{600nm} = 0.8 to 1.1, cells were collected by centrifuging at 10,000 rpm for 5 minute.
3. The collected cells were then subjected to acid shock in M9 minimal media along with 1μM monensin at different pH of 3, 4 and 5 for incubation time periods of 1h, 2h, 3h and 4h.

5.3. Internal acidification:

1. Single cell colony of both bacteria was inoculated in 250 mL M9 minimal media adjusted to pH 7 and was incubated at 37^{o}C.
2. When the optical density (OD) of inoculated culture media reached A_{600nm} = 0.8 to 1.1, cells were collected by centrifuging at 10,000 rpm for 5 minute.
3. The collected cells were then subjected to acid shock in M9 minimal media along with 20 mM sodium benzoate at different pH of 3, 4 and 5 for incubation time periods of 1h, 2h, 3h and 4h.

5.4. Preparation of cell extract:

1. Acid shocked cells were centrifuged at 10,000 rpm for 5 minute and the pelleted out cells were collected and resuspended in phosphate buffer saline.
2. Then the cells were sonicated for a total of 2 minute with 30 second interval time.
3. The sonicated sample was then centrifuged at 12,500 rpm for 25 minute and the supernatant was collected for protein estimation and enzyme assays by continuous spectroscopic rate determination.

6. Enzyme assays:

6.1 Isocitrate dehydrogenase assay:

The activity of $NADP^+$ -isocitrate dehydrogenase was assayed by measuring the reduction of $NADP^+$ at a wavelength of 340 nm. One unit of activity is that amount of isocitrate dehydrogenase catalyzing the reduction of $NADP^+$/min at 25°C (Garnak and Reeves, 1979).

$$\text{Isocitrate} + NADP^+ + Mg^{2+} \xrightarrow{\text{ICD}} \alpha\text{-ketoglutarate} + NADPH + H^+ + CO_2$$

6.1.1 Reagents required:

1. 4.6mM Isocitrate solution
2. $NADP^+$ solution
3. 0.2M Tris HCl buffer (pH 7.5)
4. 10 mM $MnCl_2$

5. Cell extract
6. Distilled water to make the assay volume to 3mL

6.1.2 Procedure:

1. 1830 µL distilled water was taken in a quartz cuvette.
2. Then 450 µL of 0.2M of tris HCl (pH7.5) was added to the assay volume.
3. Later 300 µL of 10mM $MnCl_2$ solution was added to the assay volume.
4. Then 300 µL of 5mM $NADP^+$ was added to the assay volume.
5. After that 60µL of 4.6mM isocitrate was added and mixed properly. Base line time scan was done to ensure that, no other reactions interfered with the enzyme reaction under assay.
6. Then 60 µL cell extract was added to the assay volume and time scan was performed at 340 nm in UV spectroscopy.
7. The change in absorption per minute (ΔA_{340nm}/min) was then calculated.
8. Protein was estimated by Folin Lowry method.
9. Specific activity of isocitrate dehydrogenase was calculated.

Note:
Enzyme and $NADP^+$ solution should be prepared on the day of use; enzyme solutions should be kept on ice at all times. All other solutions are prepared as stock solution and are stable for months.

6.2 Malate dehydrogenase assay:

The reaction velocity is determined by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH. One unit oxidizes one µmole of NADH per minute at 25°C and pH 7.4 under the specified conditions (Park *et al.,* 1995).

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^+$$

6.2.1 Reagents required:

1. 1M Tris HCl buffer (pH 8.2)
2. NADH solution

3. 250 mM oxaloacetate
4. cell extract
5. distilled water to make the volume of system to 3mL

6.2.2 Procedure:

1. 2280 µL distilled water was taken in quartz cuvette.
2. Then 300 µL of 1M of tris HCl (pH8.2) was added to the assay volume.
3. Then 120 µL of NADH was added to assay volume.
4. After that 120 µL of 250mM oxaloacetate was added mixed properly. Base line time scan was done to ensure that, no other reactions interfered with the enzyme reaction under assay.
5. Then 60 µL cell extract was added to the assay volume and time scan was performed at 340 nm in UV spectroscopy.
6. The change in absorption per minute (ΔA_{340nm}/min) was then calculated.
7. Protein was estimated by Folin Lowry method.
8. Then the specific activity of malate dehydrogenase was calculated.

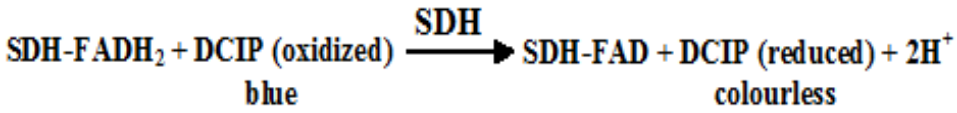
Note:

Enzyme and $NADP^+$ solution should be prepared on the day of use; enzyme solutions should be kept on ice at all times. All other solutions are prepared as stock solution and are stable for months.

6.3 Succinate dehydrogenase assay:

The enzymatic activity of succinate dehydrogenase can be measured by monitoring the reduction of an artificial electron acceptor, 2,6-dichlorophenolindophenol (DCIP), in a reaction mixture. The addition of sodium azide blocks the normal path of electrons in the electron transport chain thereby preventing the transfer of electrons from cytochrome a_3 to oxygen, the final acceptor in the chain. Instead the cytochromes and coenzyme Q pass electrons to DCIP. One molecule of DCIP is reduced by one molecule of succinate. The reduction of DCIP was measured spectroscopically since the oxidized form of the dye is blue and the reduced form is colorless (Spencer and Guest, 1973).

The reaction is summarized as:



6.3.1 Reagents required:

1. 125 mM Potassium phosphate buffer (pH 7.4)
2. 1M Sodium succinate
3. Sodium azide
4. Diclorophenolindophenol (DCIP)
5. Cell extract
6. Distilled water to make the volume of system to 3 mL.

6.3.2 Procedure:

1. 2400 µL of 125mM potassium phosphate buffer (pH7.4) was taken in a glass cuvette.
2. Then 60 µL cell extract was added to the assay volume.
3. After that a pinch of sodium azide was added to the assay volume.
4. Then after proper mixing, the system was incubated for 15 minute at room temperature.
5. Then 45µL of 1 M sodium succinate was added to the assay volume.
6. Immediately after that, a pinch of DCIP was added and mixed properly, which turned the solution blue.
7. The absorbance was measured at 600 nm in UV/visible spectrocopy.
8. The change in absorption per minute (ΔA_{600nm}/min) was calculated.
9. Protein was estimated by Folin Lowry method.
10. Specific activity of malate dehydrogenase was calculated.

Note:

Cell extract should be prepared on the day of use; enzyme solutions should be kept on ice at all times. All other solutions are prepared as stock solution and are stable for months.

6.4 Glucose-6-phosphate dehydrogenase assay:

(Banerjee and Fraenkel, 1972)

One unit of activity is the amount of enzyme required to catalyze the reduction of 1 µmole of $NADP^+$ per min at 25°C.

$$\text{G-6-P} + \text{NADP}^+ + \text{H}^+ \xrightarrow{\text{G6PD}} \text{6-Phosphoglucono-δ-lactone} + \text{NADPH}$$

6.4.1 Reagents required:

1. 1 M Tris HCl buffer (pH 8.2)
2. 0.1 M $MgCl_2$
3. 5 mM $NADP^+$ solution
4. 0.1 M Glucose-6-phosphate
5. Cell extract
6. Distilled water to make the volume of system to 3mL.

6.4.2 Procedure:

1. 2100 µL distilled water was taken in a quartz cuvette.
2. 300 µL of 0.2M of tris HCl (pH8.2) was added to the assay volume.
3. Later 300 µL of 0.1M $MgCl_2$ solution was added to the assay volume.
4. Then 60 µL of 5 mM $NADP^+$ was added to the assay volume.
5. After that 300 µL of 0.1 M glucose-6-phosphate was added and mixed properly. Base line time scan was done to ensure that, no other reactions interfered with the enzyme reaction under assay.
6. Then 60µL cell extract was added to the assay volume and time scan was performed at 340 nm in UV spectroscopy.
7. The change in absorption per minute (ΔA_{340nm}/min) was then calculated.
8. Protein was estimated by Folin Lowry method.
9. Specific activity of glucose-6-phosphate dehydrogenase (G6PD) was calculated.

Note:

Enzyme and $NADP^+$ solution should be prepared on the day of use; enzyme solutions should be kept on ice at all times. All other solutions are prepared as stock solution and are stable for months.

7. Calculating enzymes activity

7.1 Formula for calculating enzyme activity:

μmol/min/ml = (ΔA_{340nm}/min) X (3 /6.2) X (1/extract volume) X (df)

Where,

1. ΔA_{340nm}/min = Change in absorbance per minute
2. 3 =Volume (in milliliters) of assay
3. df = Dilution factor
4. 6.22 = Millimolar extinction coefficient for ß-NADPH at 340 nm
5. Extract volume = Volume (in milliliter) of enzyme used
   (Smeets and Muller, 1971).
   Note: for DCIP, 20.2 $mM^{-1}cm^{-1}$ extinction coefficient was used.

7.2 Specific activity of enzymes:

One unit of activity is the amount of enzyme required to catalyze the reduction of 1 μmole of $NADP^+$ per min at 25°C. Specific activity is expressed in μM/min/mg of protein.

μM/min/mg = (Enzyme activity) / (protein concentration)

8. Protein estimation by Folin Lowry:

8.1 Principle:

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen(s) with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteau phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids.

8.2 Reagents required:

1. Folin reagent: Folin and Ciocalteu's phenol reagent
2. 1% Sodium tartarate: Na_2tatarat.$2H_2O$
3. 0.5% Copper sulphate: $CuSO_4.5H_2O$
4. 0.1 M NaOH
5. Na_2CO_3 (2%)
6. Bovine Serum albumin (BSA)

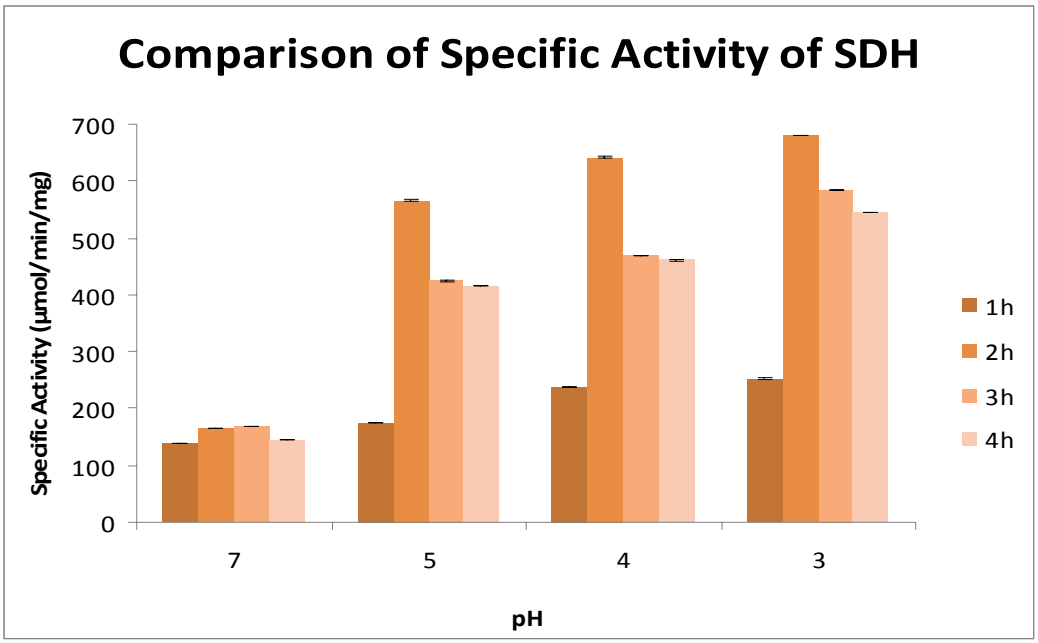
8.3 Procedure:

48 ml anhydrous sodium carbonate (2%) in sodium hydroxide (0.1M) + 1ml 0.5% copper sulphate: $CuSO_4.5H_2O$ + 1 ml 1% sodium tartarate: Na_2tatarat.$2H_2O$ were mixed immediately prior to use and was labeled as solution A. Then various aliquots of BSA were prepared with increasing concentration of protein and to them were added prepared unknown with 0.5 mL of cell extract. Then 5 mL of solution A was added to all the tubes. 0.5mL of Folin and Ciocalteu's phenol reagent was added to all tubes. Then the tubes were incubated in dark for 30 min at room temperature. Readings were noted at 540 nm against H_2O as blank. A standard graph was plotted from which the concentration of protein in cell extract was determined.

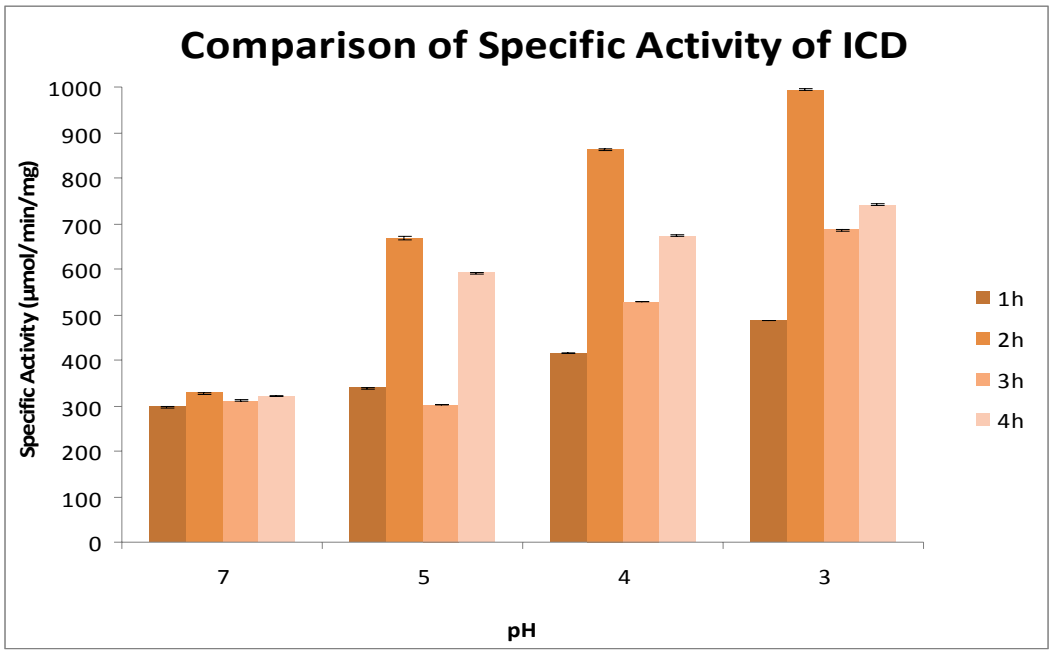
RESULTS

It's not what you know, but what you learn after you know it, that counts.

External acidification of *E. coli* DH5α:

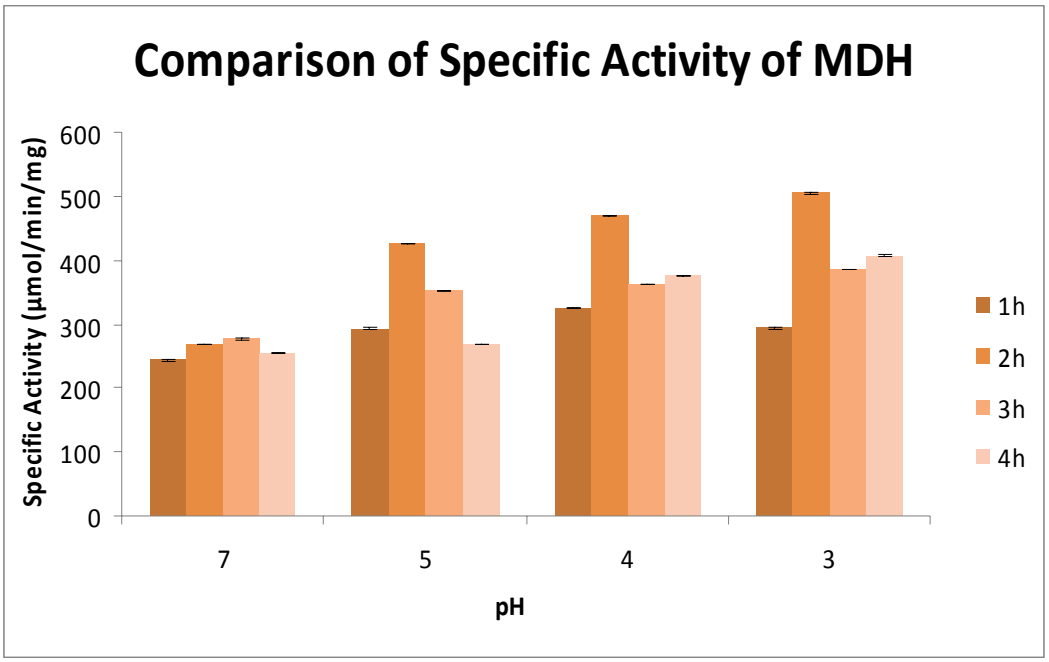


[A]

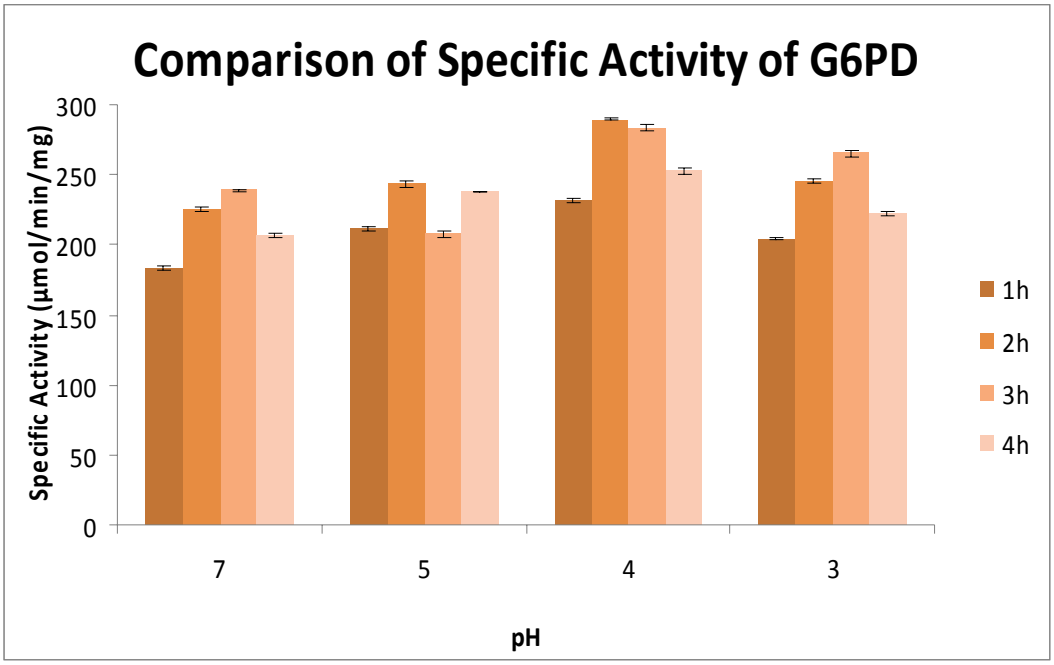


[B]

Figure 7: Comparison of specific activity of [A] SDH, [B] ICD from *E. coli* DH5α at different time periods upon exposure to different low pH (acidic shock). pH 7 is used as control (Mean ± SD for 3 values).



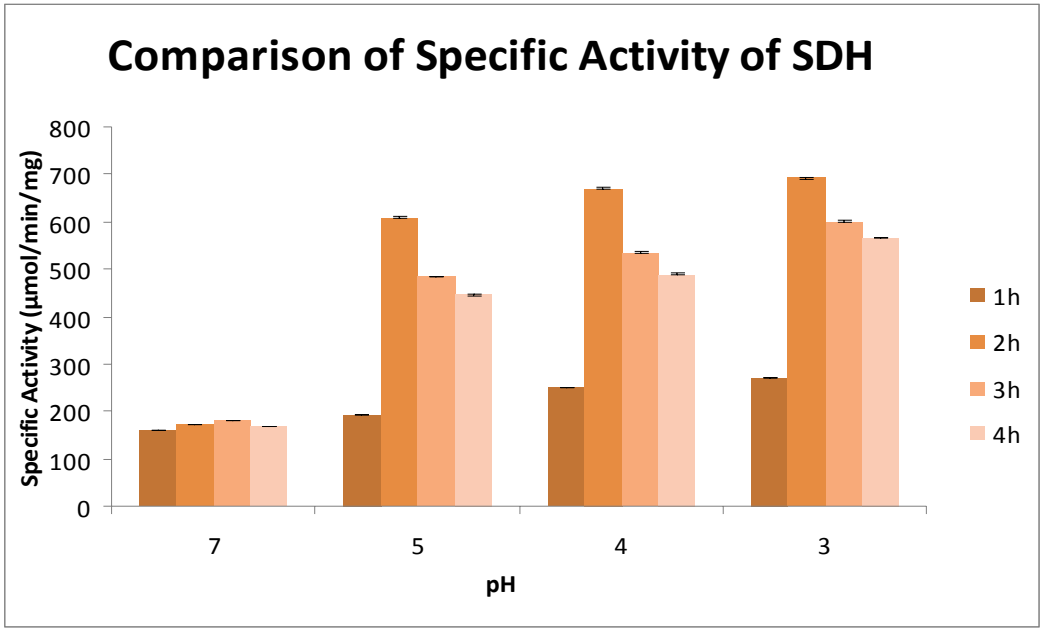
[A]



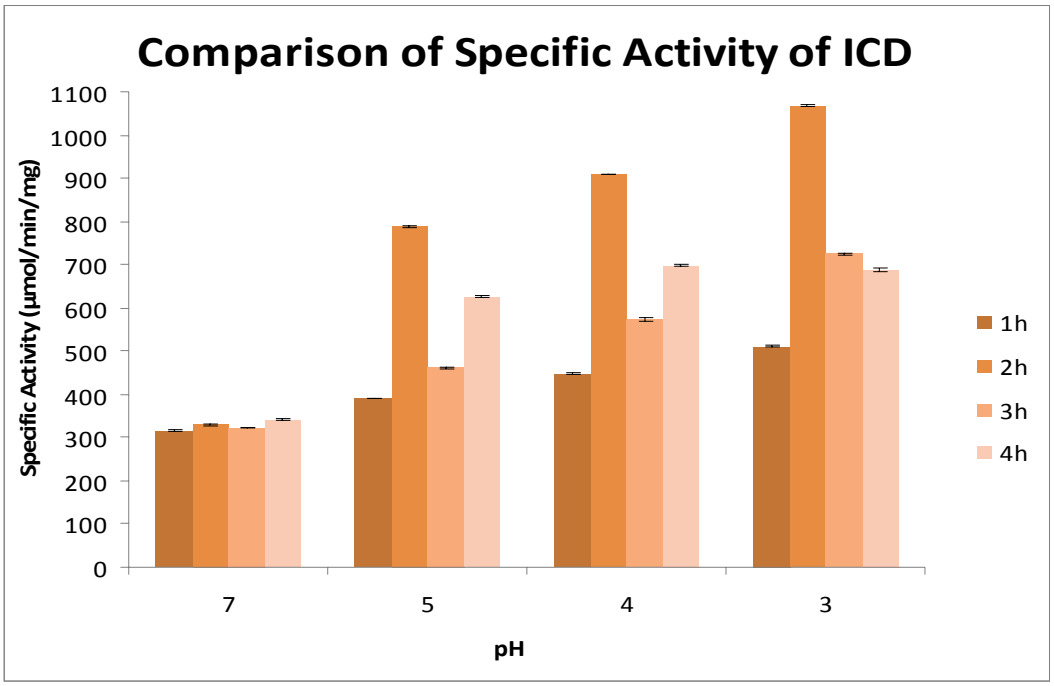
[B]

Figure 8: Comparison of specific activity of [A] MDH, [B] G6PD from *E. coli* DH5α at different time periods upon exposure to different low pH (acidic shock). pH 7 is used as control (Mean ± SD for 3 values).

External acidification of *E. coli* W3110:

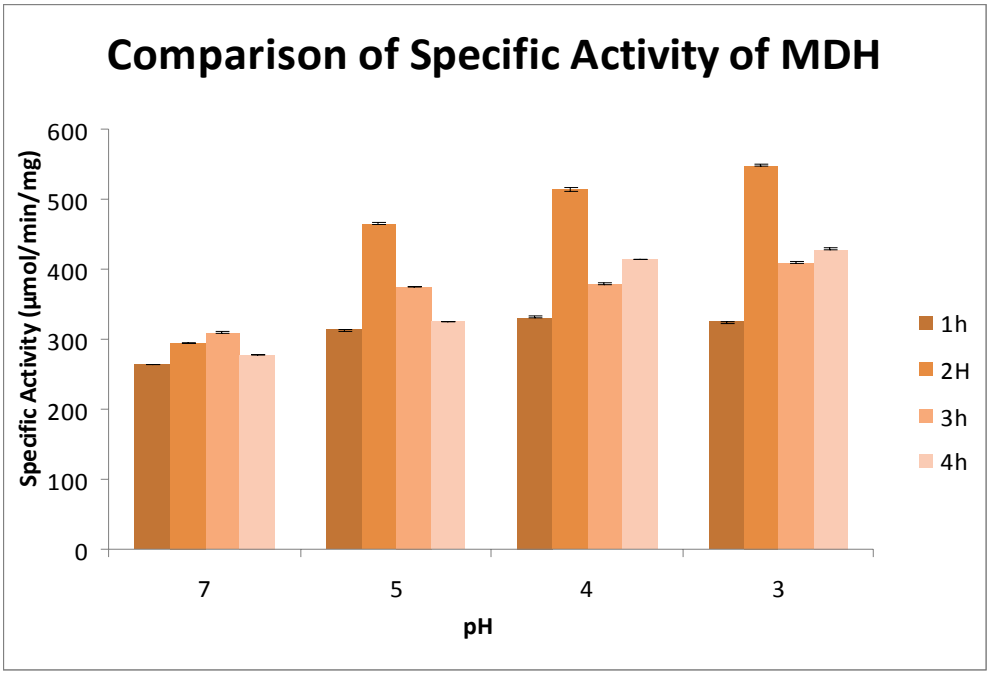


[A]

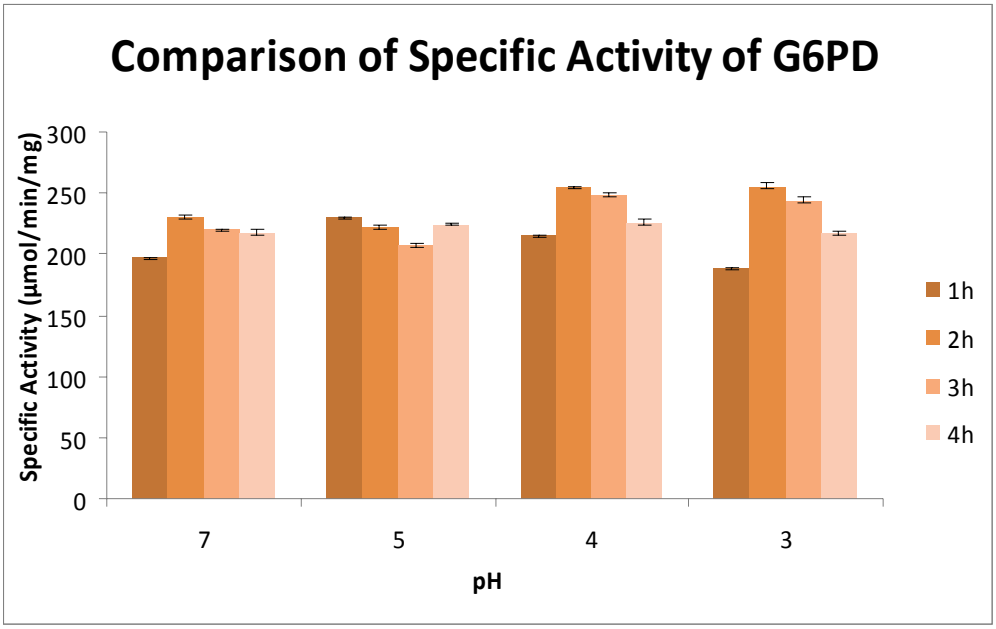


[B]

Figure 9: Comparison of specific activity of [A] SDH, [B] ICD from *E. coli* W3110 at different time periods upon exposure to different low pH (acidic shock). pH 7 is used as control (Mean ± SD for 3 values).



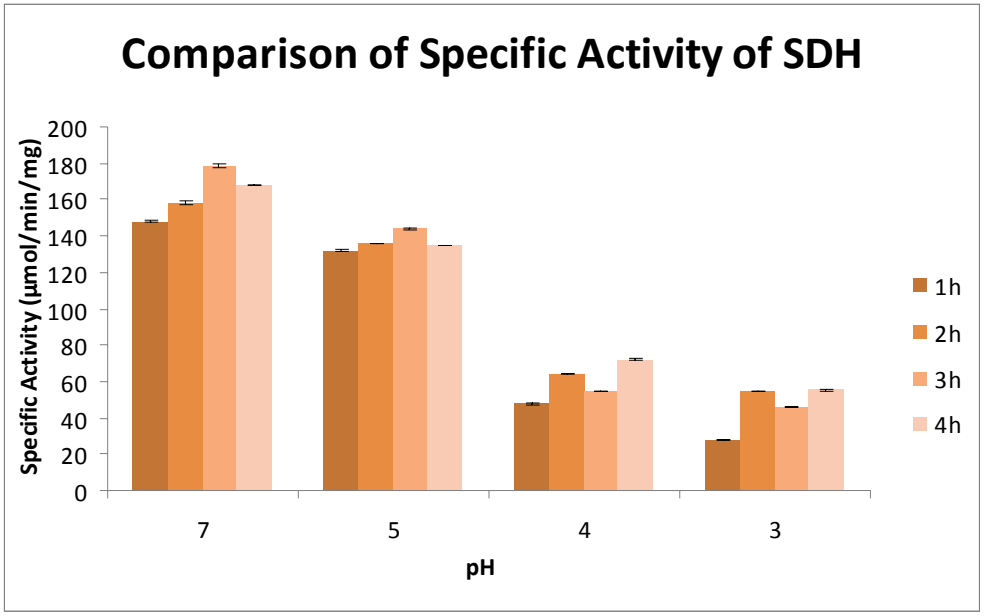
[A]



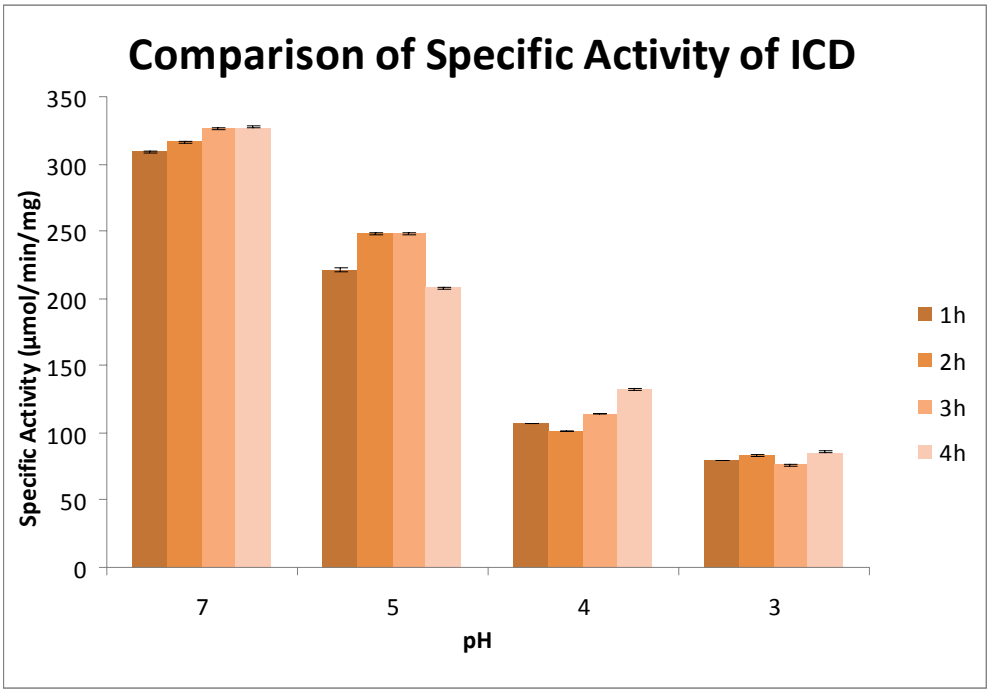
[B]

Figure 10: Comparison of specific activity of [A] MDH, [B] G6PD from *E. coli* W3110 at different time periods upon exposure to different low pH (acidic shock). pH 7 is used as control (Mean ± SD for 3 values).

Internal acidification of *E. coli* DH5α:

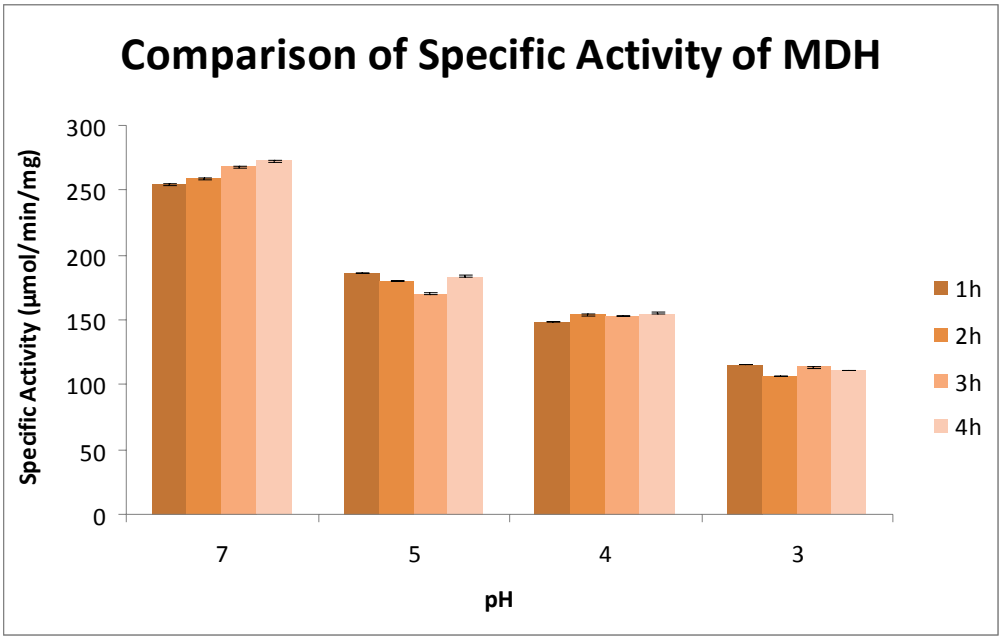


[A]

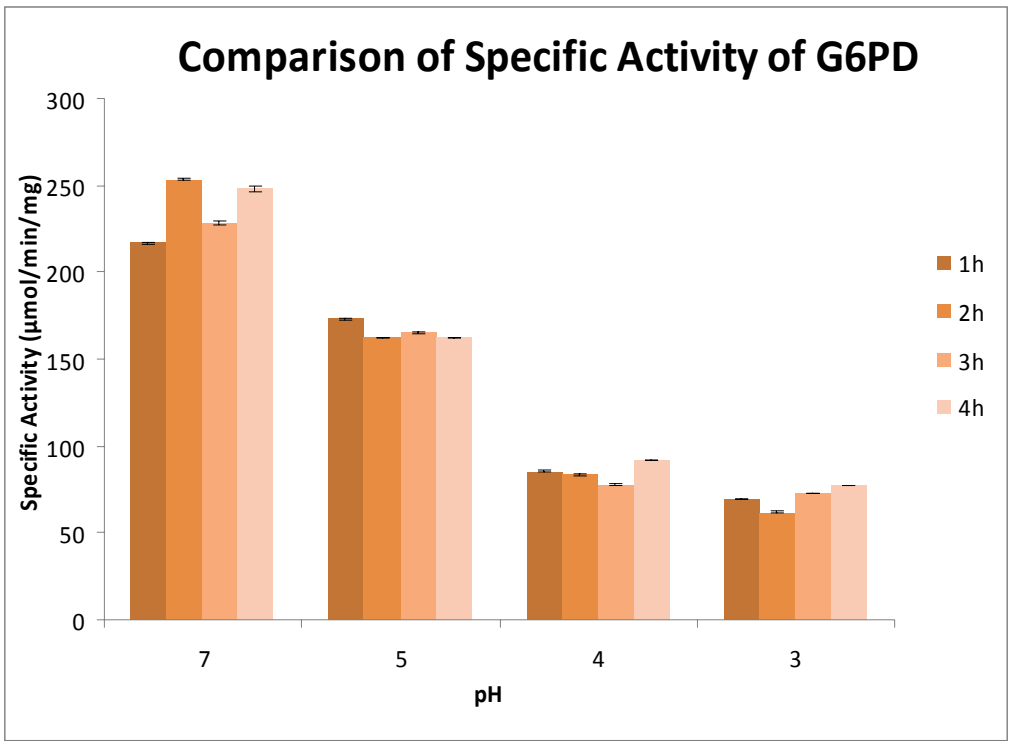


[B]

Figure 11: Comparison of specific activity of [A] SDH, [B] ICD from *E. coli* DH5α at different time periods upon exposure to different low pH (acidic shock) using sodium benzoate as an acid permeant (20mM). pH 7 is used as control (Mean ± SD for 3 values).



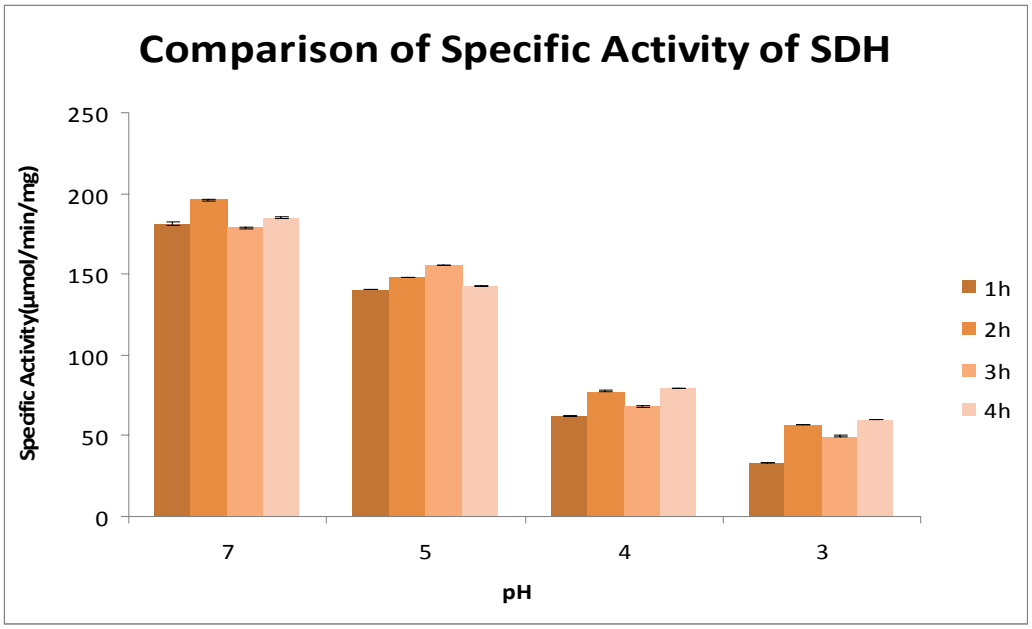
[A]



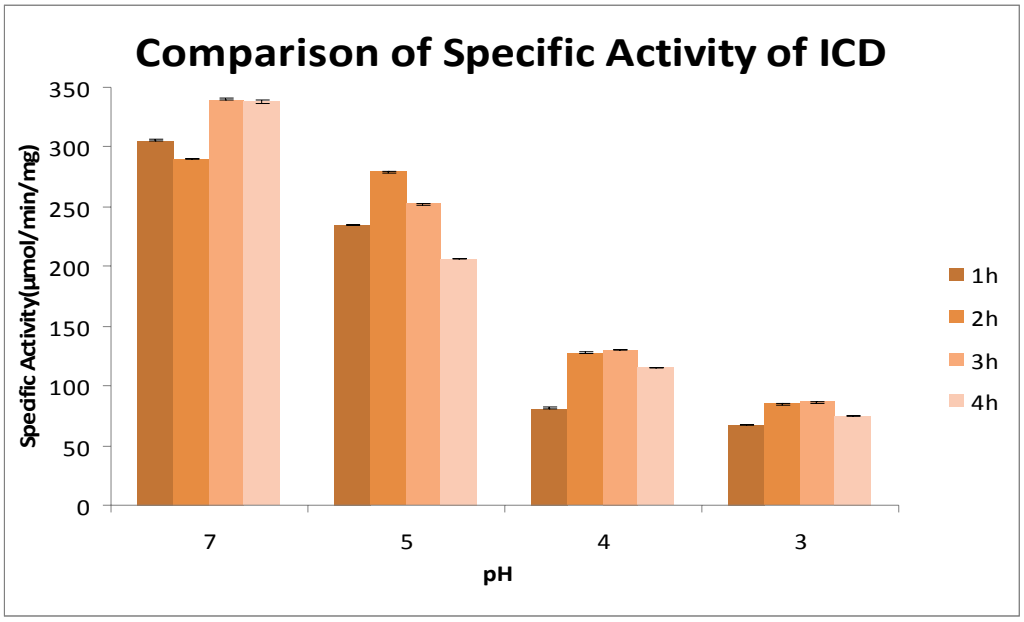
[B]

Figure 12: Comparison of specific activity of [A] MDH, [B] G6PD from *E. coli* DH5α at different time periods upon exposure to different low pH (acidic shock) using sodium benzoate as an acid permeant (20mM). pH 7 is used as control (Mean ± SD for 3 values).

Internal acidification of *E. coli* W3100:

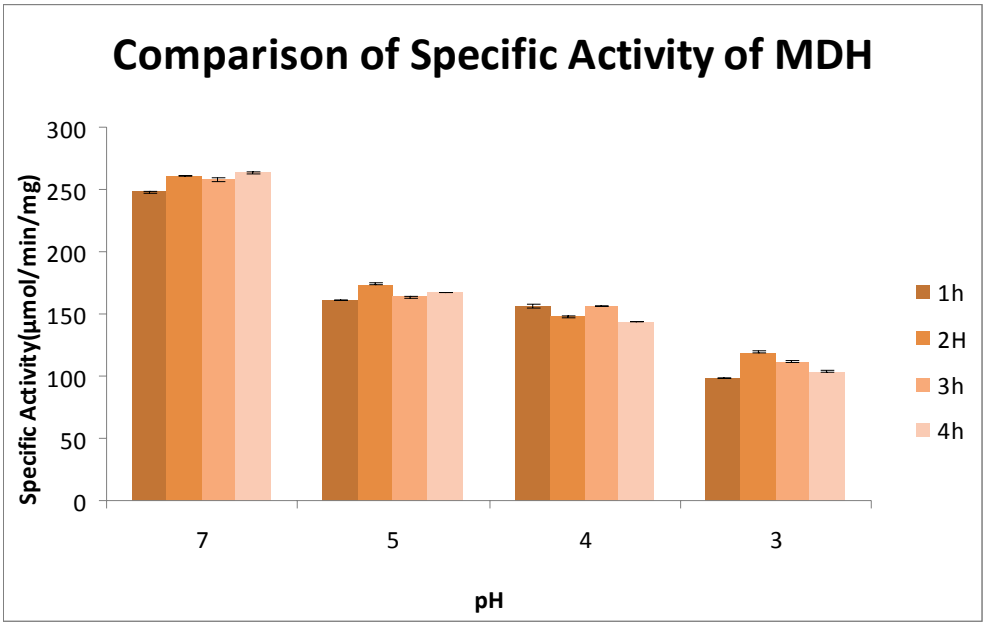


[A]

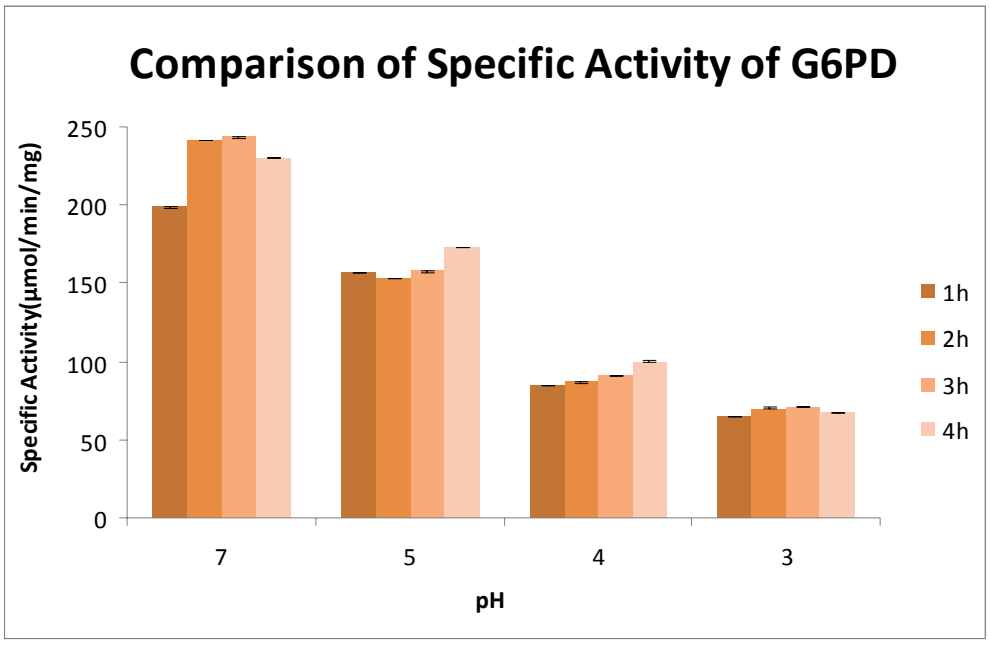


[B]

Figure 13: Comparison of specific activity of [A] SDH, [B] ICD from *E. coli* DH5α at different time periods upon exposure to different low pH (acidic shock) using sodium benzoate as an acid permeant (20mM). pH 7 is used as control (Mean ± SD for 3 values).



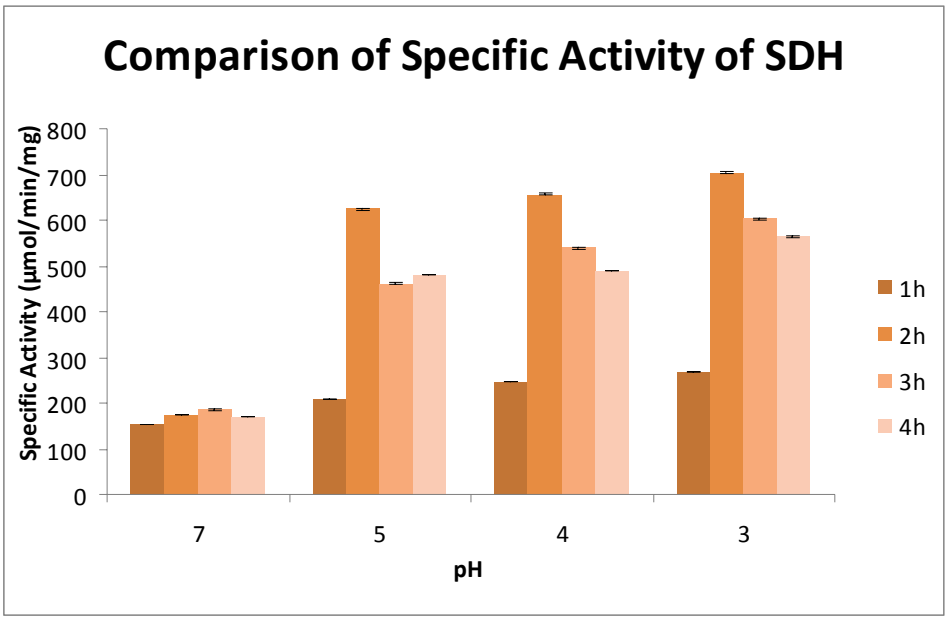
[A]



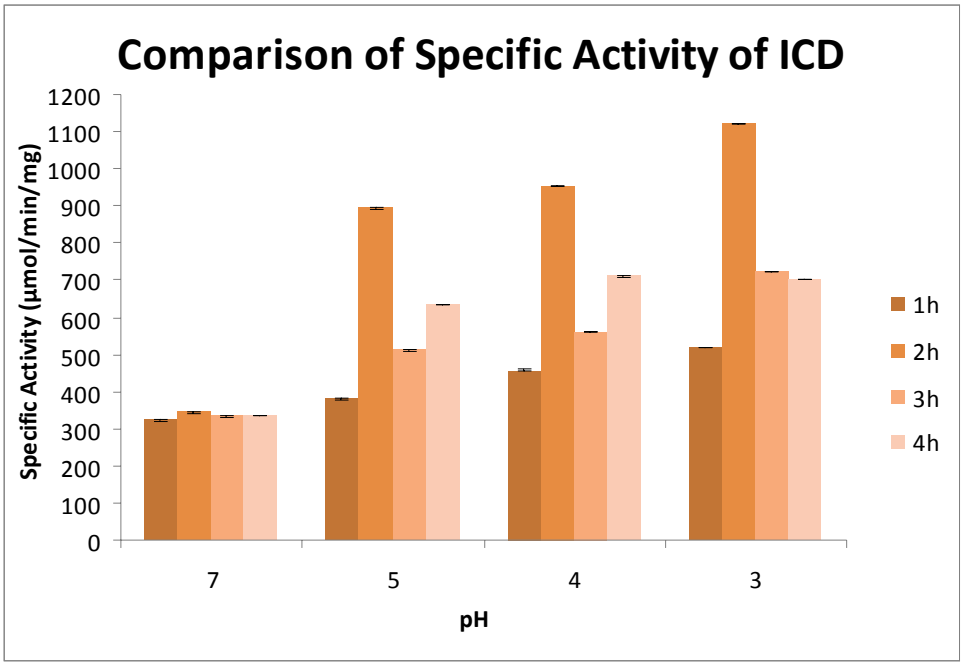
[B]

Figure 14: Comparison of specific activity of [A] MDH, [B] G6PD from *E. coli* DH5α at different time periods upon exposure to different low pH (acidic shock) using sodium benzoate as an acid permeant (20mM). pH 7 is used as control (Mean ± SD for 3 values).

External acidification of *E. coli* DH5α along with monensin:

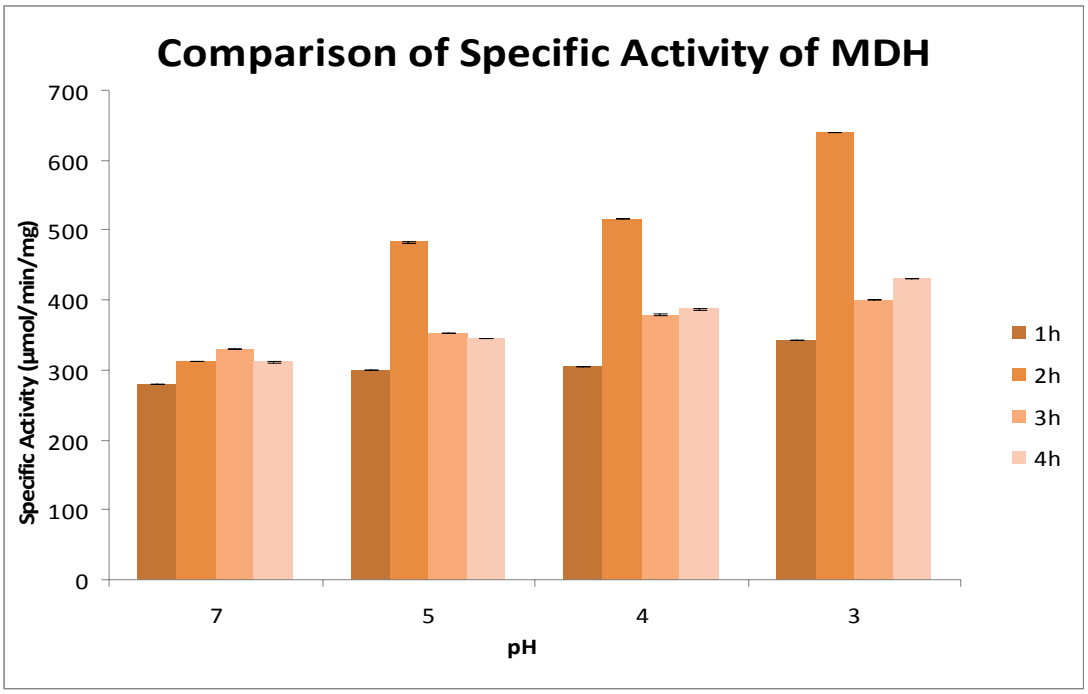


[A]

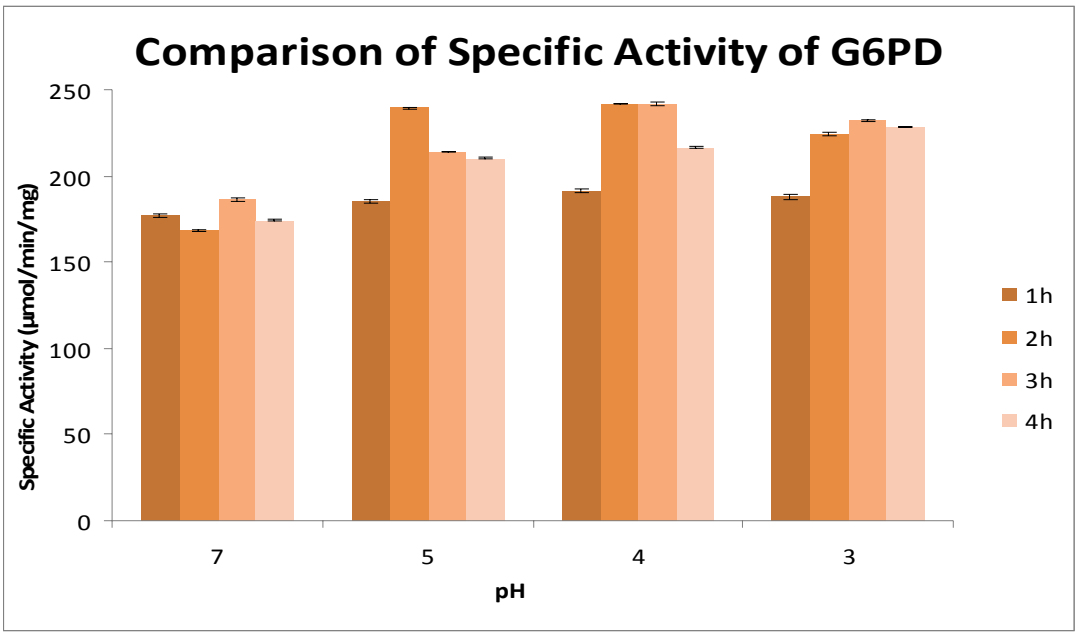


[B]

Figure15:Comparison of specific activity of [A] SDH, [B] ICD from *E. coli* DH5α at different time periods upon exposure to different low pH (acidic shock) using monensin as an uncoupler (1µM). pH 7 is used as control (Mean ± SD for 3 values).



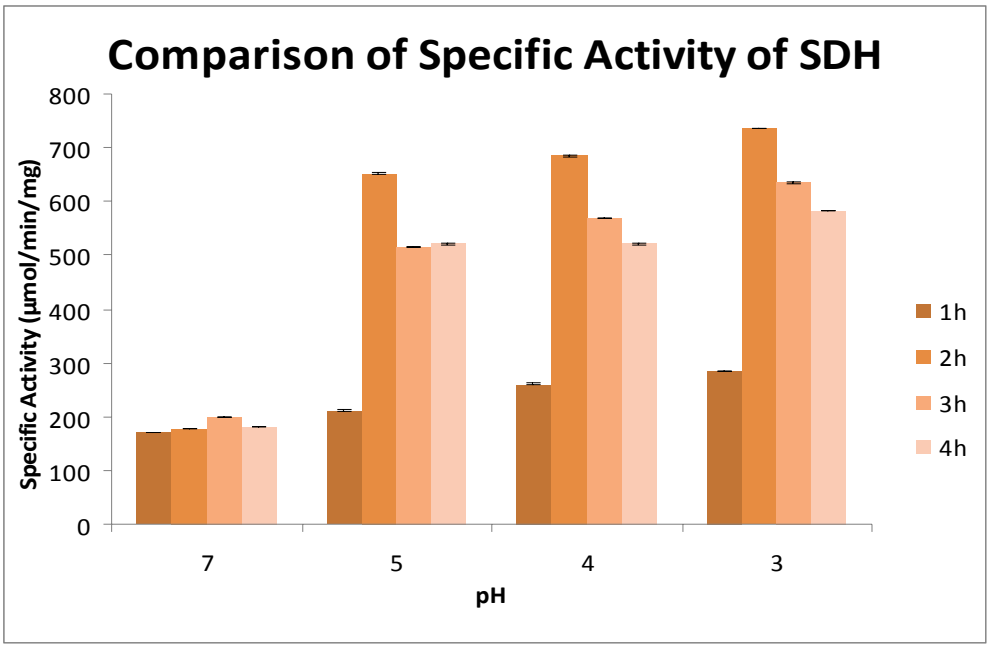
[A]



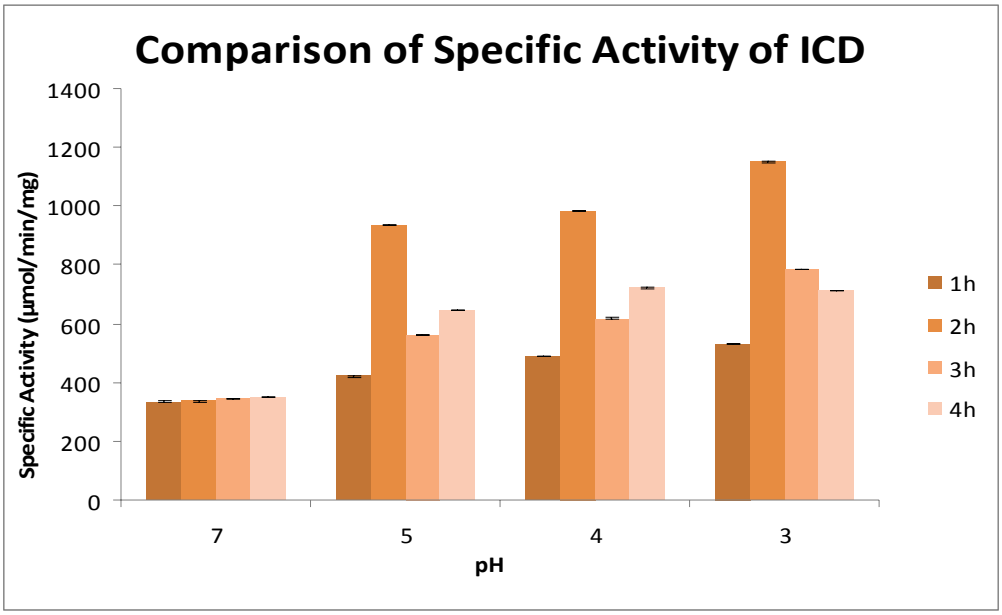
[B]

Figure 16: Comparison of specific activity of [A] MDH, [B] G6PD from *E. coli* DH5α at different time periods upon exposure to different low pH (acidic shock) using monensin as an uncoupler (1µM). pH 7 is used as control (Mean ± SD for 3 values).

External acidification of *E. coli* W3100 along with monensin:

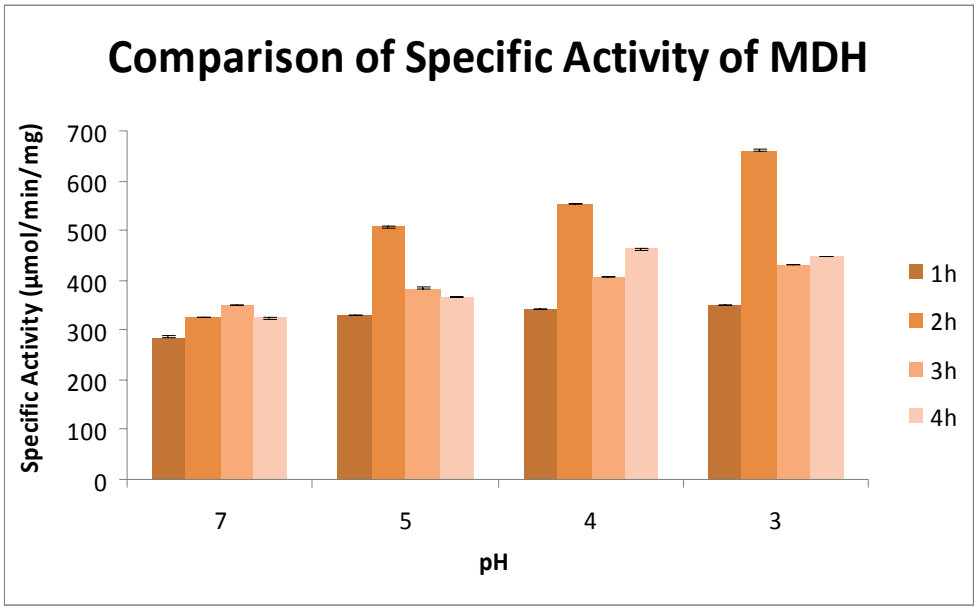


[A]

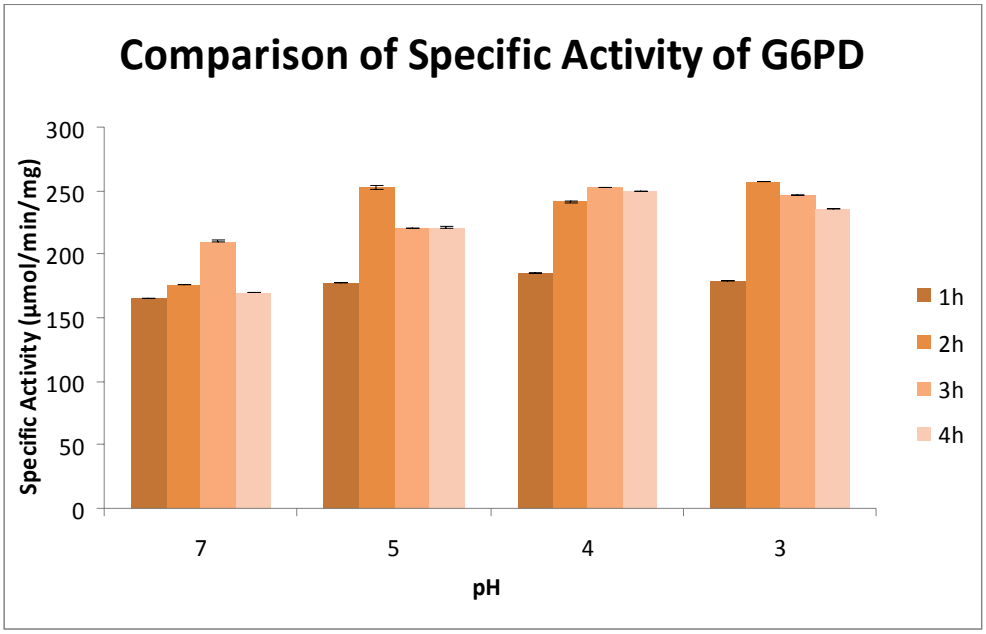


[B]

Figure 17: Comparison of specific activity of [A] SDH, [B] ICD from *E. coli* W3110 at different time periods upon exposure to different low pH (acidic shock) using monensin as an uncoupler (1µM). pH 7 is used as control (Mean ± SD for 3 values).



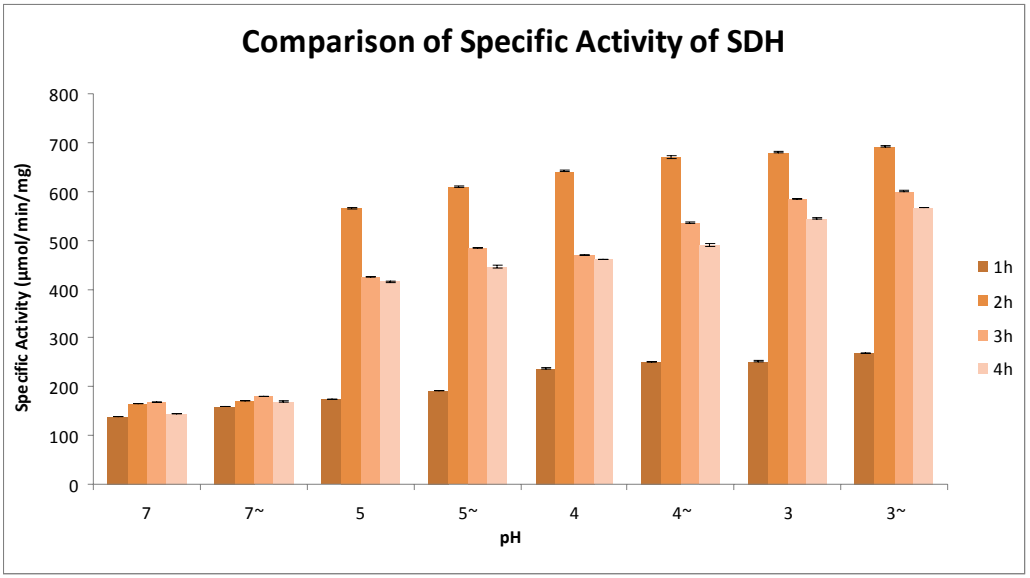
[A]



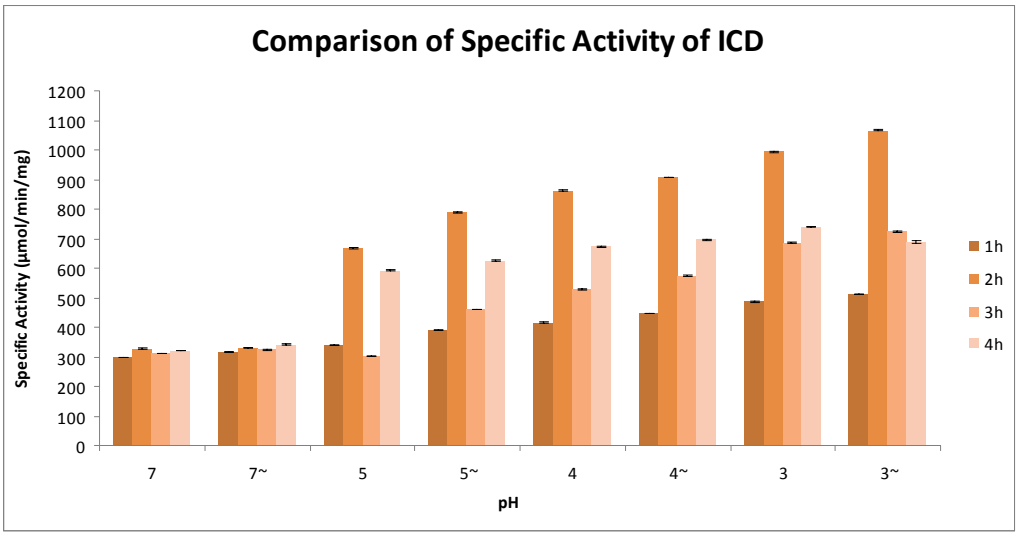
[B]

Figure 18: Comparison of specific activity of [A] MDH, [B] G6PD from *E. coli* W3110 at different time periods upon exposure to different low pH (acidic shock) using monensin as an uncoupler (1µM). pH 7 is used as control (Mean ± SD for 3 values).

Comparison of specific activities of dehydrogenases when exposed to external acidification of *E. coli* DH5α and *E. coli* W3110:



[A]

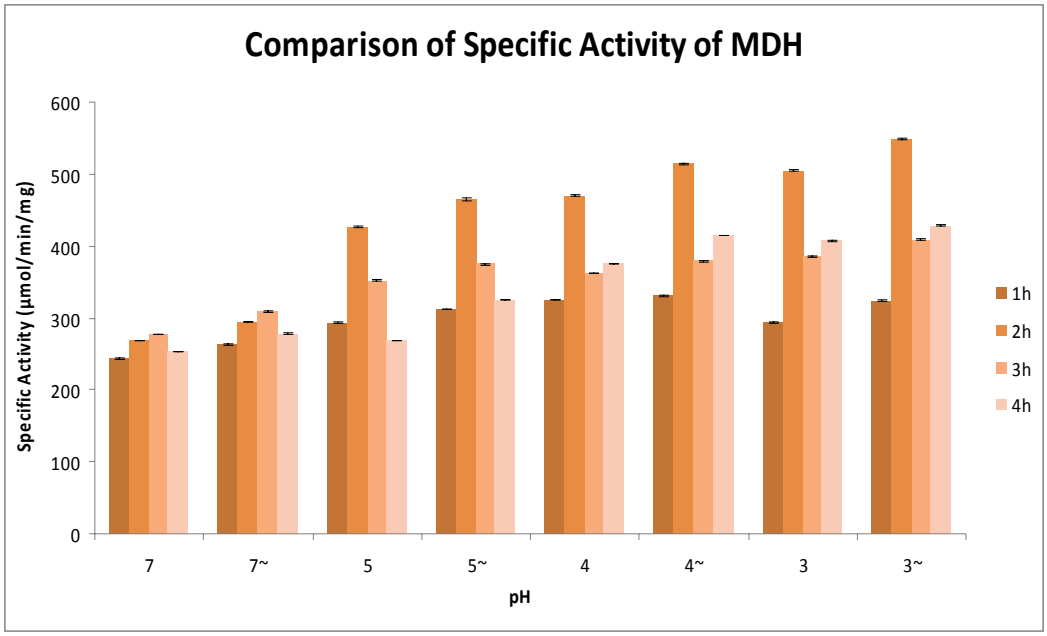


[B]

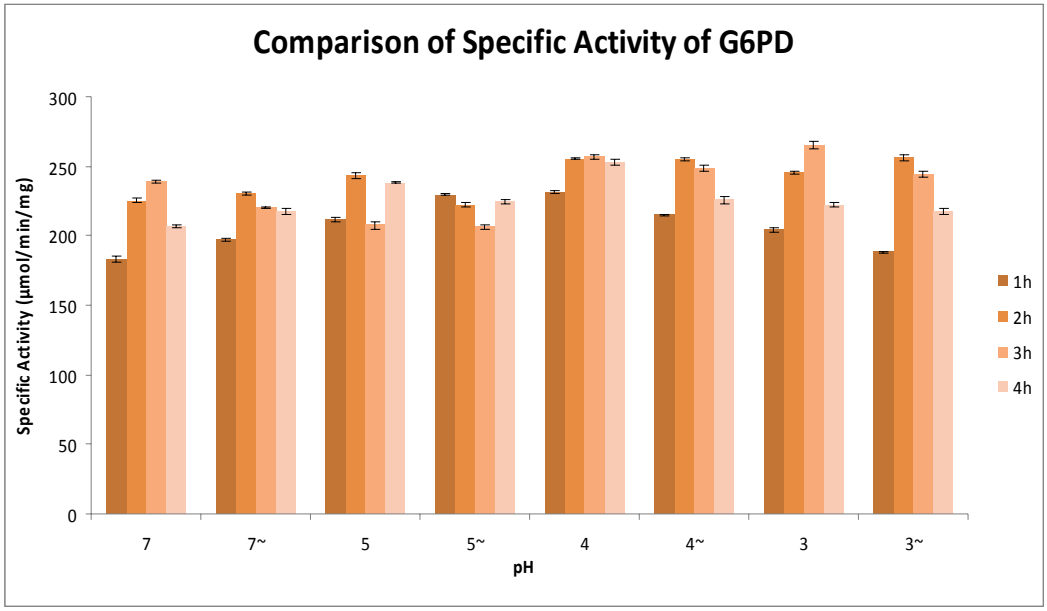
Figure 19: Comparison of specific activity of [A] SDH, [B] ICD following exposure to external acidification in *E. coli* DH5α and *E. coli* W3110 at different time periods (Mean ± SD for 3 values).

pH External acidification only in *E. coli* DH5α.

pH˜ External acidification in *E. coli* W3110.



[A]



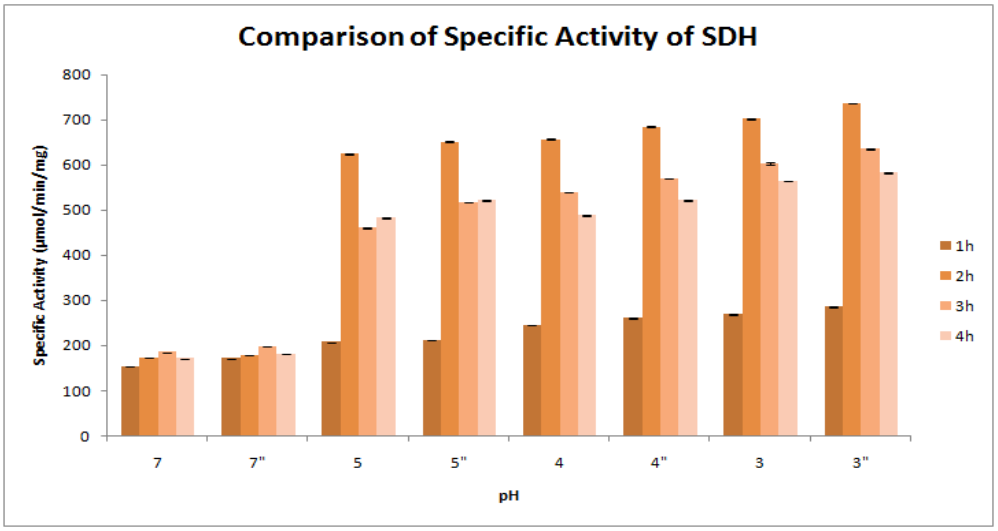
[B]

Figure 20: Comparison of specific activity of [A] MDH, [B] G6PD following exposure to external acidification in *E. coli* DH5α and *E. coli* W3110 at different time periods (Mean ± SD for 3 values).

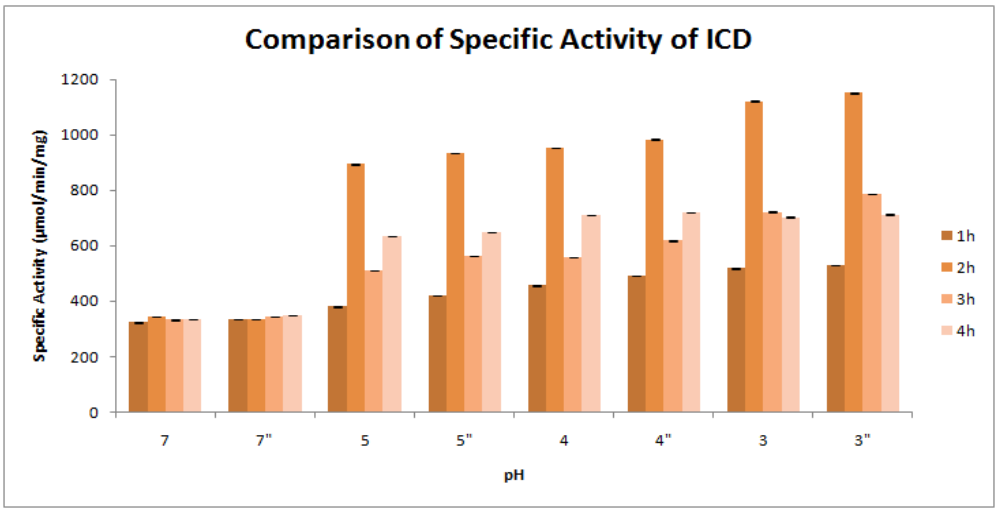
pH External acidification only in *E. coli* DH5α.

pH˜ External acidification in *E. coli* W3110.

Comparison of specific activities of dehydrogenases when exposed to external acidification along with monensin of *E. coli* DH5α and *E. coli* W3110:



[A]

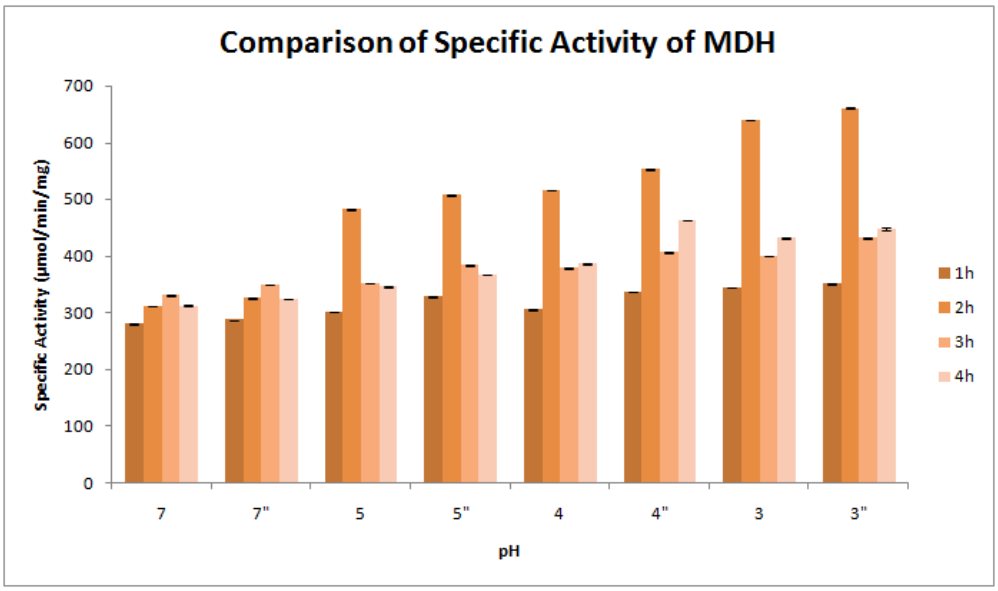


[B]

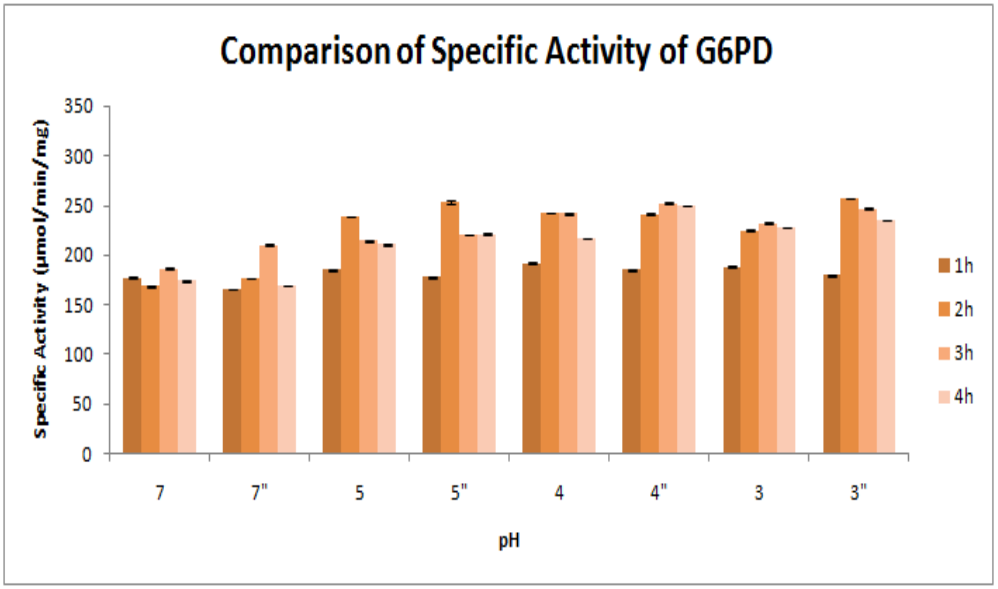
Figure 21: Comparison of specific activity of [A] SDH, [B] ICD following exposure to external acidification and monensin in *E. coli* DH5α and W3100 at different time periods (Mean ± SD for 3 values).

pH External acidification along with monensin in *E. coli* DH5α.

pH* External acidification along with monensin in *E. coli* W3110.



[A]



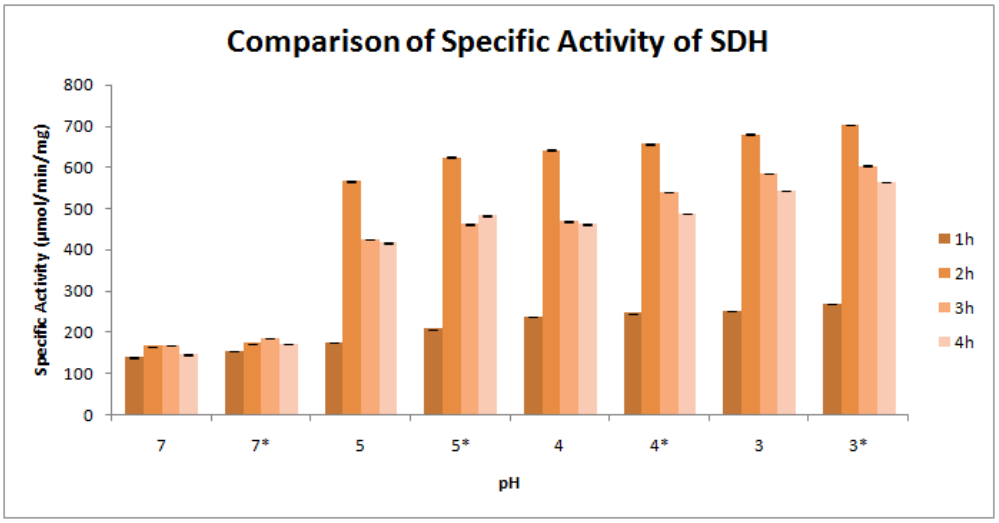
[B]

Figure 22: Comparison of specific activity of [A] MDH, [B] G6PD following exposure to external acidification and monensin in *E. coli* DH5α and W3100 at different time periods (Mean ± SD for 3 values).

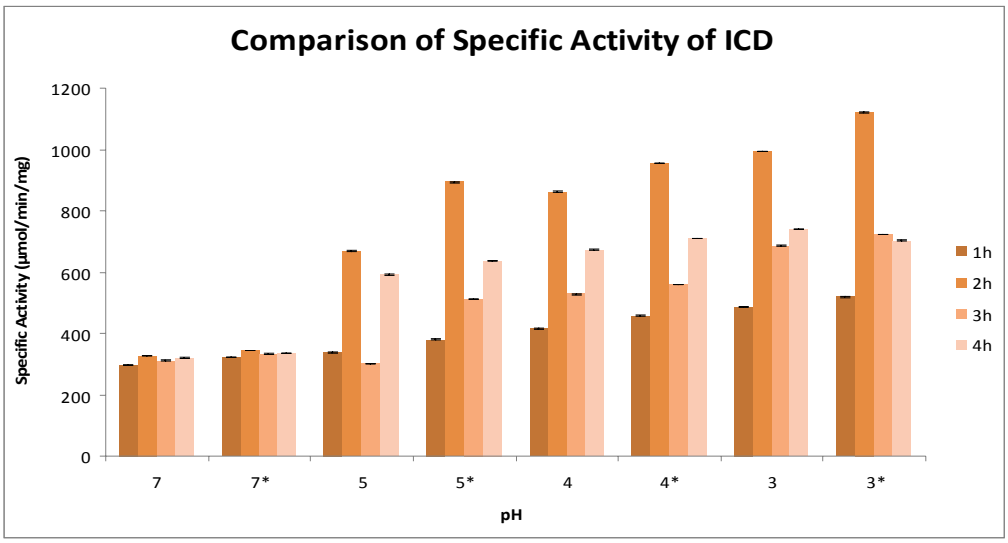
pH External acidification along with monensin in *E. coli* DH5α.

pH* External acidification along with monensin in *E. coli* W3110.

Comparison of specific activities of dehydrogenases when exposed to external acidification and external acidification along with monensin of *E. coli* DH5α:



[A]

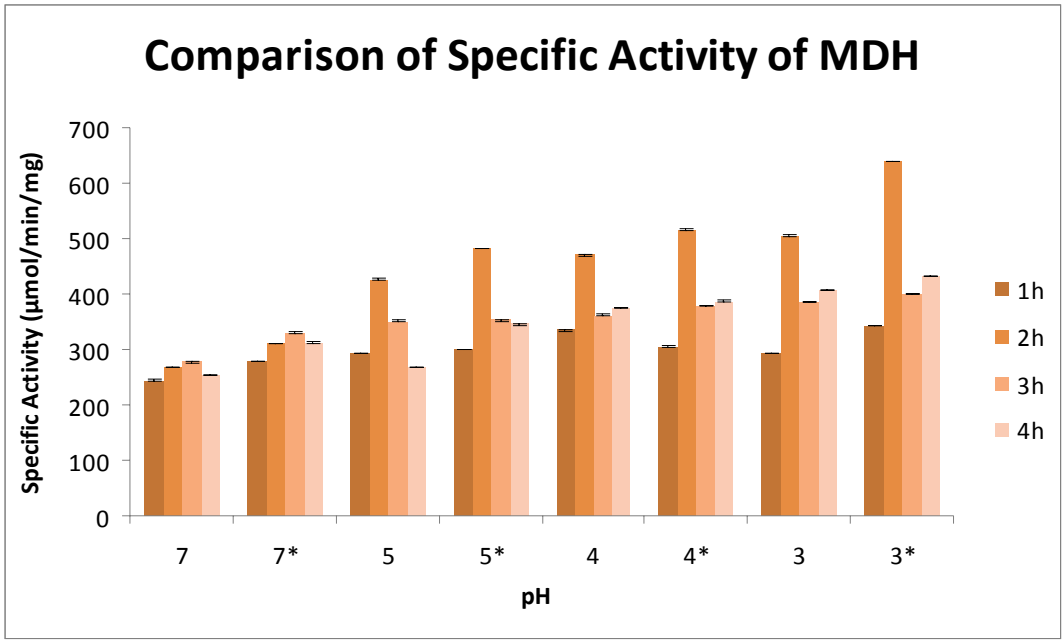


[B]

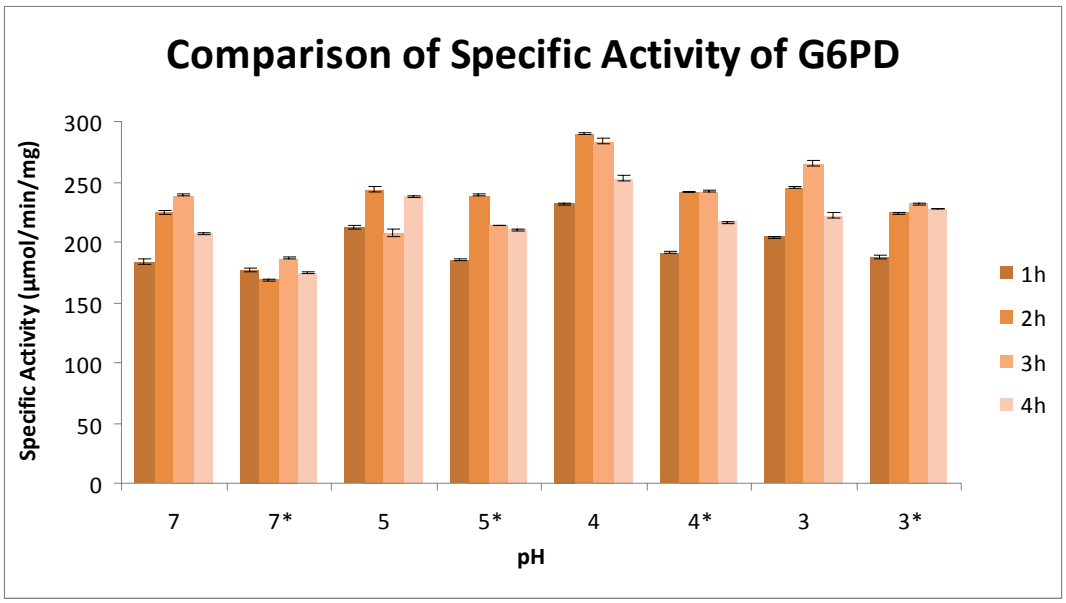
Figure 23: Comparison of specific activity of [A] SDH, [B] ICD following exposure to external acidification and external acidification along with monensin in in *E. coli* DH5α at different time periods (Mean ± SD for 3 values).

pH External acidification only in *E. coli* DH5α.

pH* External acidification along with monensin in *E. coli* DH5α.



[A]



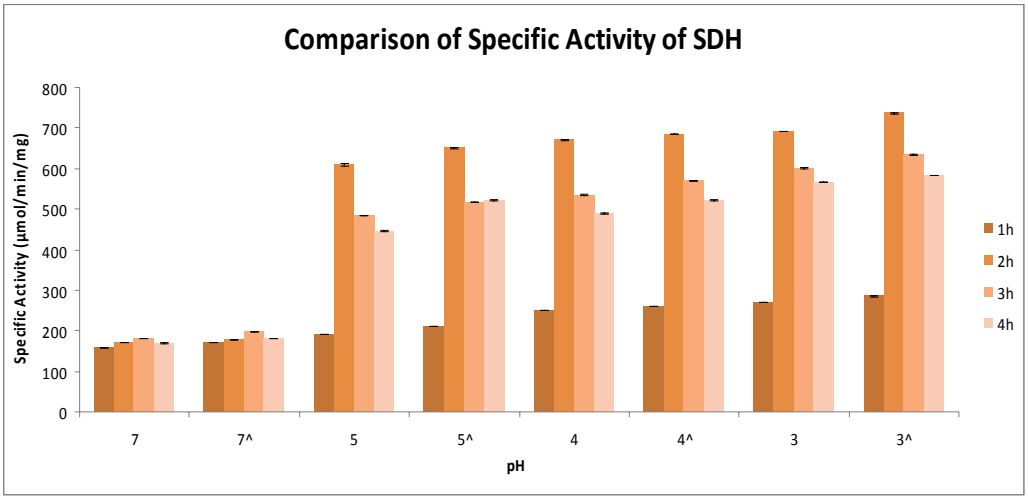
[B]

Figure 24: Comparison of specific activity of [A] MDH, [B] G6PD following exposure to external acidification and external acidification along with monensin in *E. coli* DH5α at different time periods (Mean ± SD for 3 values).

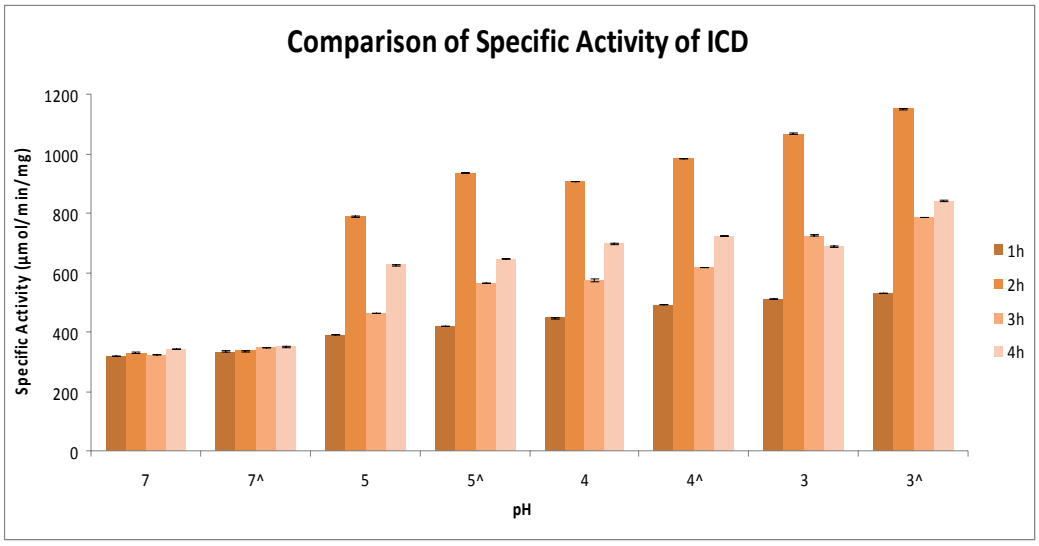
pH External acidification only in *E. coli* DH5α.

pH* External acidification along with monensin in *E. coli* DH5α.

Comparison of specific activities of dehydrogenases when exposed to external acidification and external acidification along with monensin of *E. coli* W3110:



[A]

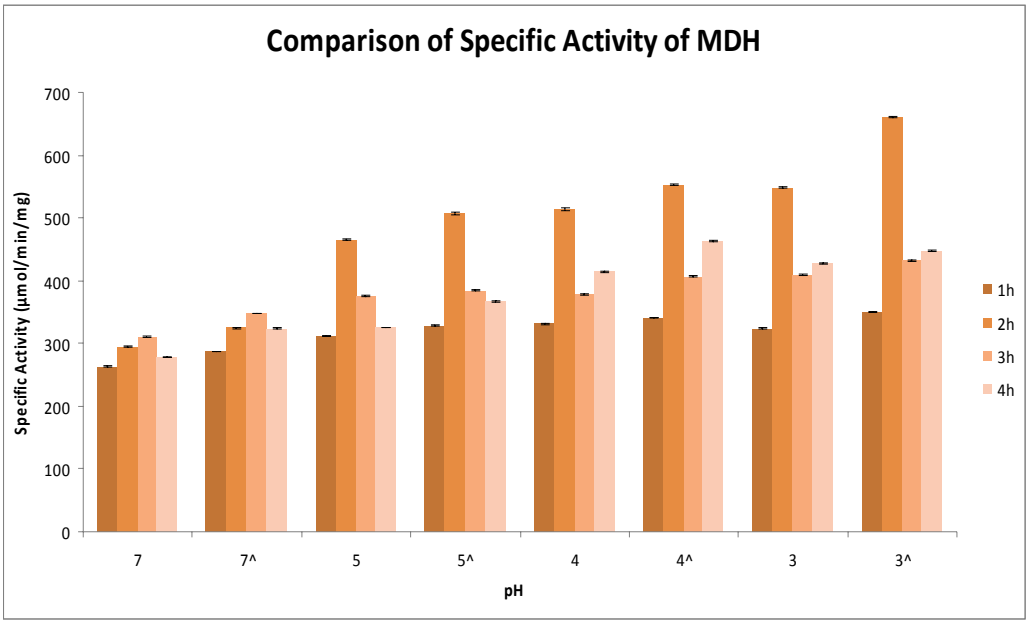


[B]

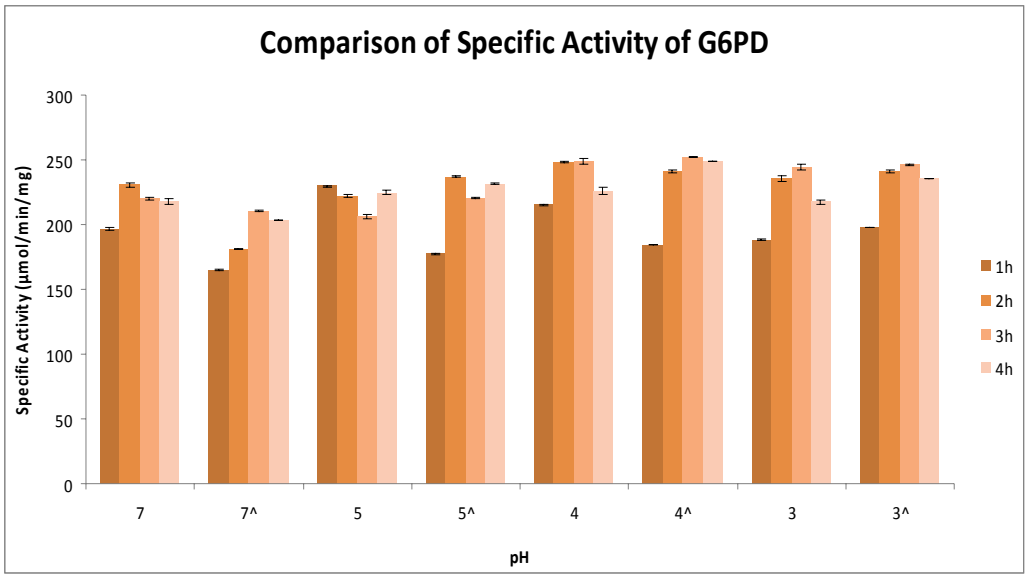
Figure 25: Comparison of specific activity of [A] SDH, [B] ICD following exposure to external acidification and external acidification along with monensin in *E. coli* W3110 at different time periods (Mean ± SD for 3 values).

pH External acidification only in *E. coli* W3110.

pH^ External acidification along with monensin in *E. coli* W3110.



[A]



[B]

Figure 26: Comparison of specific activity of [A] MDH, [B] G6PD following exposure to external acidification and external acidification along with monensin in *E. coli* W3110 at different time periods (Mean ± SD for 3 values).

pH External acidification only in *E.coli* W3110.

pH^ External acidification along with monensin in *E.coli* W3110.

DISCUSSION AND CONCLUSION

Knowledge is the true organ of sight, not the eyes.

Most studies on pH homeostasis have been focused on those aspects where bacterial growth and survival have an impact on human health and economics, i.e., survival and growth in food, effect on the oral cavity, gastric transit, and intracellular survival. The pH surrounding cells or cell aggregates is the main environmental factor that strongly determines their growth and metabolism. In order to colonize their mammalian hosts, both commensal and pathogenic enteric bacteria must survive passage through the low pH environment of the stomach. Humans secrete approximately 2.5 liter of gastric juice each day, generating a fasting gastric pH of 1.5, which increases to pH between 3.0 and 5.0 during feeding (Hill, 2002). Enteric pathogens must survive this level of pH for at least 2 h, the average emptying time for the stomach, before reaching the intestine (Texter, E. C., Jr 1968).

Bacteria have developed different ways to withstand stressful situations, such as a decrease in the external pH. Florence *et al*. found that different induced acid resistant systems in *E. coli* depend on whether oxidative or fermentative metabolism is occurring. These systems are, change in cellular envelope to decrease ionic permeability, induction of DNA repair, chaperones, development of ionic pumping system and majorly the amino acid decarboxylase system (glutamate decarboxylase, arginine decarboxylase, and lysine decarboxylase). While our understanding of the mechanisms used by neutrophiles to grow or survive in low pH environments has increased, much remains to be determined. The present work is an investigation of the otherwise unexplored role of dehydrogenases in the acid adaptation of a neutrophile.

E. coli cells when subjected to acid shock environments were shown to undergo dramatic changes in specific activities of ICD, SDH, and MDH while the activity of G6PD remained unchanged. The extent of increased acid tolerance was affected by the time of adaptation and adaptation pH. Among the various adaptation times and adaptation pH tested, exposure of *E. coli* to acid at pH 3 for 2 h resulted in an increased acid tolerance for both the conditions including external acidification and external acidification along with monensin.

On external acidification:

Much of the success of bacteria depends on their ability to survive and thrive in adverse conditions. External acidification of the environment of *E. coli* results in a net inflow of protons resulting in acidification of cytoplasm. pH homeostasis is an energy dependent process, which restores the cytoplasmic pH to near neutrality. The pH of the cell cytoplasm is a critical parameter controlling a variety of cellular processes.

The effect of a lowered extracellular pH on *E. coli* activates various pH homeostasis mechanisms which restore the pH of the cytoplasm to near neutral. Pumping out protons from the cell is one of the many mechanisms that undo the effect of a lowered extracellular pH. Dehydrogenases when convert their substrate into its product, generate reducing power which on getting recycled at the ETC results in extrusion of protons out of the cell. In the present work this expected result was confirmed by an increase in the specific activities of SDH, ICD and MDH on external acidification. An increase in the specific activity suggests a higher flux of metabolites through the TCA cycle along with the recycling of the reducing power at the ETC. Moreover, the results in the present work are in accord with the work of Kannan *et al.* where these authors have, on a transcriptomic level, observed an up-regulation of succinate dehydrogenase on rapid external acidification of *E. coli* W3110.

G6PD is a key enzyme of the pentose phosphate pathway and its substrate G-6-P can be channeled along this pathway, or via glycolysis into the TCA cycle. Hence, under certain situations competition for the channeling of common substrate, G-6-P, between these pathways plays an important role. At low pH of 3, 4, and 5 channeling of G-6-P through glycolysis into TCA cycle was higher than when compared to pentose phosphate pathway in both *E. coli* DH5α and *E. coli* W3100. Evidence of such channeling came from the following observation -a rise in specific activities of SDH, ICD and MDH of the TCA cycle when compared to no noteworthy change in the specific activity of G6PD. This suggests that, at low pH, metabolites are not diverted from the central metabolic pathway to other sub-branching pathways. In this way nearly all the reducing power generated through the central metabolic pathway may be used for pumping out protons through the ETC, thereby helping in maintenance of pH homeostasis of the cytoplasm.

On internal acidification:

In the gastrointestinal tract, enteric bacteria are subjected to acid stress from strong acid (HCl) as well as bacterial fermentation products such as acetic, propionic, and butyric acids, which are membrane-permeant weak acids (Lin *et al*., 1996; Polen *et al*., 2003). The cytoplasm of *E. coli* may be acidified without changing the external pH, for example, by addition of membrane permeant weak acid like sodium benzoate, which is used as a food preservative. The action of sodium benzoate is dependent on the pH value which involves diffusion of the lipophilic, undissociated acid molecule through the plasma membrane into the cytoplasm where it dissociates into charged anions and protons resulting in cytoplasmic acidification. A fall by two to three folds in the specific activities of all four dehydrogenases was observed as a consequence of cytoplasmic acidification in *E. coli* suggesting that these enzymes may have an optimum pH range of near neutral to alkaline.

On external acidification along with monensin:

The proton motive force, generated across the inner membrane of *E. coli*, is a summation of electrical and chemical potential energies [pmf = $\Delta\Psi$ + ΔpH]. Monensin, a monovalent ion-selective ionophore, facilitates the transmembrane exchange of principally sodium ions for protons (Russell and Strobel, 1989). The outer surface of the ionophore-ion complex is composed largely of nonpolar hydrocarbon, which imparts a high solubility to the complexes in nonpolar solvents. In biological systems, these complexes are freely soluble in the lipid components of membranes and, presumably, diffuse or shuttle through the membranes from one aqueous membrane interface to the other. Monensin binds to Na^+ ions (from NaCl in the growth medium) in the extracellular space and shuttles them into the cytoplasm of the bacterium. In this way it disrupts the electrical potential generated across its membrane and thus, acts as an uncoupler of oxidative phosphorylation. Since the electrical potential has been nullified by an uncoupler, to regain its actual pmf the bacterial cell enhances its chemical potential by increased expulsion of H^+ ions through the ETC (Russell, 1987). If, however, the external pH is lowered there will be a need to further increase the H^+ ion expulsion. Reducing power generated within a cell with its recycling along the ETC expel H^+ ions. Therefore, in both the scenarios (with or without acid shock), it is expected that the rate of recycling of the reducing power between the TCA cycle and the ETC may increase. In the present work this expected result was confirmed by

an increase in the specific activities of SDH, ICD and MDH on external acidification along with the use of monensin when compared to external acidification alone. This further increase in specific activities suggests a higher rate of recycling of reducing power between the TCA cycle and the ETC and also an increment in the metabolic flux through the TCA cycle.

E. coli excretes acetate due to increased pyruvate flux from glucose transport by the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (Chang et al., 1999). *E. coli* W3100 *pta* mutant is defective in phosphotransacetylase, the first enzyme of the acetate pathway, sparing acetyl-CoA molecules to be fluxed through the TCA cycle which otherwise would have been excreted as acetate molecules in *E. coli* DH5α.

PTA (phosphotransacetylase) is a key enzyme of the acetate pathway and in a number of bacteria its substrate is acetyl-CoA which can, in certain metabolic situations, be channeled along this pathway. In normal situations acetyl-CoA is channeled preferably through the tricarboxylic acid cycle. Hence, in certain situations a competition between the acetate pathway and the tricarboxylic acid cycle for the common substrate, acetyl-CoA may take place. At low pH conditions of 3, 4, and 5 along with an uncoupler -monensin, acetyl-CoA channeling through the TCA cycle was higher in *E. coli* W3110 when compared to *E. coli* DH5α. The evidence of such channeling came from the following observation - a rise in specific activities of SDH, ICD, and MDH of the TCA cycle in *E. coli* W3110 when compared to that in *E. coli* DH5α. This is in agreement with the mutant nature of the strain *E. coli* W3110. This suggests, in *E. coli* W3110, a high metabolic flux through the TCA cycle results in a higher recycling of the reducing power between the TCA cycle and the ETC resulting in enhanced pumping of protons as compared to *E. coli* DH5α. This manifests improved acid tolerance in *E. coli* W3110 than *E. coli* DH5α.

The specific activity of G6PD remained unchanged in both *E. coli* W3100 and *E. coli* DH5α stains indicating that there may be a normal flux of metabolites through the pentose phosphate pathway. Here again, results indicate that metabolites are preferably routed through the central metabolic pathway leading to higher recycling of reducing power at the ETC resulting in enhanced pumping of protons, thereby, contributing to the maintenance of pH homeostasis.

The human stomach produces gastric juice having a pH range of 1.8 to 2.5, which increases to between pH 3.0 and 5.0 during feeding and 2 h is the average emptying time for the stomach (Texter, E. C., Jr 1968). Documentation of highest activities of all tested dehydrogenases, viz. SDH, ICD and MDH in the 2nd hour of time adaptation to external pH under both the conditions including external acidification and external acidification along with monensin may point towards increased pH homeostasis as one of the many survival strategies possessed by *E. coli*, in its successful colonization of the human gastrointestinal tract. The results of this study may indicate that acid shocked *E. coli* for a period of two hours can survive for a sustained period.

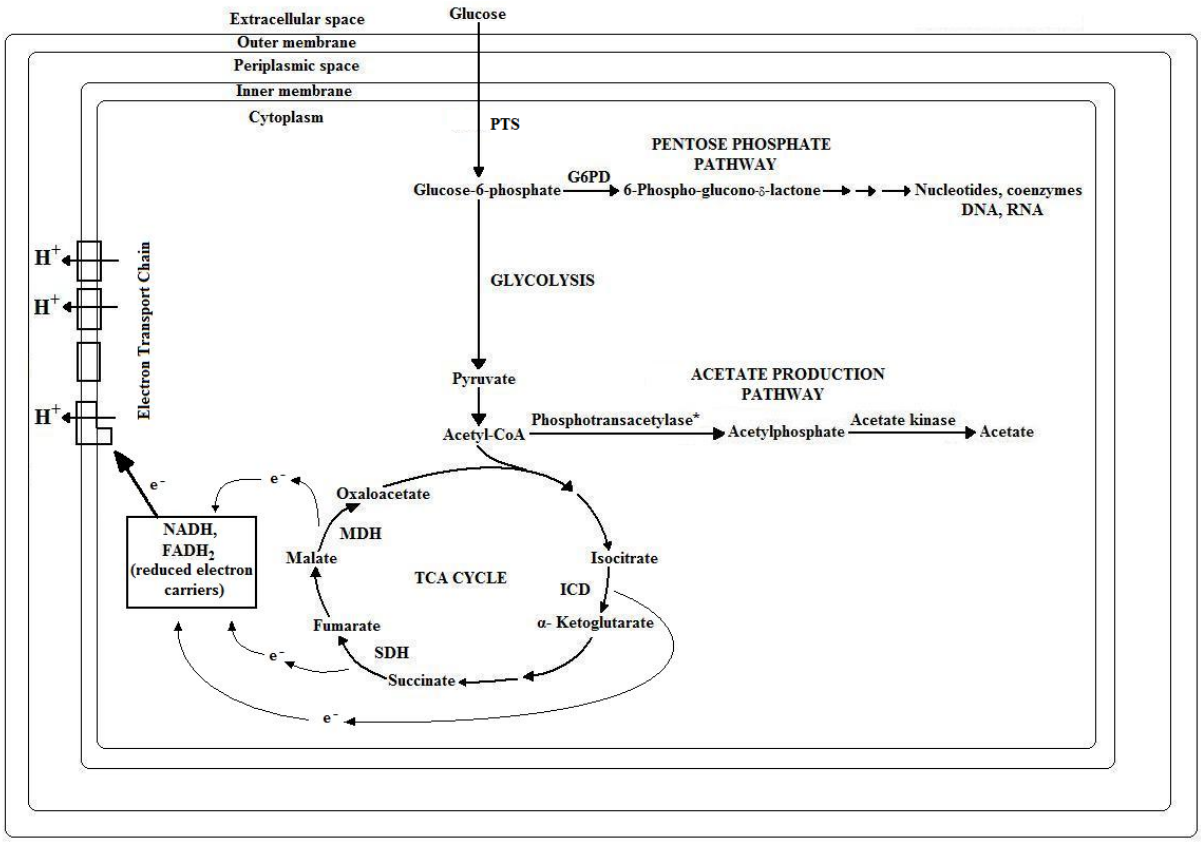
SUMMARY

To attempt to 'cover the content' would limit students to simply learning facts without the ability to apply their knowledge to solve new problems. This would encourage the mistaken belief that science is about learning the correct answer rather than about innovation, creativity and inquiry.

The extreme acidity (~pH 2.0) within the stomach presents a formidable hurdle for bacteria whose primary niche is in the lower intestinal tract of warm-blooded animals. The adaptation to changes in pH can thus be seen to significant aspects of the pathogenesis of the organism. Low pH is detrimental to microbes due to the denaturation of essential macromolecules, like proteins, and the acidification of the cytoplasm that disrupts enzymatic reactions and membrane potentials. Bacteria regulate their cytoplasmic pH within relatively narrow limits despite their adoption of environments that vary widely in pH. *E. coli* preserves the integrity of proteins and nucleic acids present in the cytoplasm by maintaining cytoplasmic pH at approximately pH 7.4 to 7.8. Most bacteria maintain a relatively neutral intracellular pH and control is generally achieved by various types of mechanism viz. the activity of ion transport systems which facilitate the entry or exit of proton.

This study examined the activities of SDH, ICD, MDH and G6PD in acid shocked cells of *Escherichia coli* DH5α and *Escherichia coli* W3110 subjected to pH 3, 4, and 5 by different types of acidification, viz. external (using 0.1N HCl), external along with the monensin (1μM) and cytoplasmic (using 20 mM sodium benzoate), for time intervals of 1, 2, 3 and 4 h where then compared with their activities at pH 7 (taken as control).

The results show that an exposure to acidic environment (pH 3, 4 and 5) for a short period of time increased the activities of these dehydrogenases in all types of acidification except cytoplamic acidification used in the current study. On cytoplasmic acidification the activities of all dehydrogenases decreased at pH 3, 4 and 5. On external acidification along with monensin, activities of dehydrogenases increased further as compared to external acidification alone. Cells exposed to pH 3 for 2 h had the highest acid tolerance on external acidification with or without monensin. It was also found that activity of G6PDremained unchanged at low pH. This result suggested that, in a low pH environment, metabolic flux in *E. coli* increases through TCA cycle and remains unaffected through the pentose phosphate pathway. This increase in metabolic flux through TCA cycle, during oxidative phosphorylation, cause electrons from NADH or $FADH_2$ to pass onto O_2 through the ETC located in the plasma membrane of the microorganism, leading to the pumping of protons out of the cytoplasm and thus maintaining pH homeostasis. The results of this study highlighted new insights into activities of SDH, ICD, MDH and G6PD coupled to the electron transport chain by the reducing power, as yet another system possessed by *Escherichia coli* as an armor against harsh acidic environments.



* This enzyme and hence the pathway is absent in *Escherichia coli* W3110.

Figure 27: Proposed mechanism for acid shock generated acid tolerance in *Escherichia coli*. A low pH external environment caused accumulation of protons in the bacterial cell cytoplasm. In such a situation the activities of dehydrogenases namely, SDH, ICD and MDH were found to increase in *E. coli* DH5α and *E. coli* W3110. This suggests that these dehydrogenases of the TCA cycle may be actively involved in expelling these excess protons from the cytoplasm. The activity of G6PD remained unaltered in both the strains. This suggests that to prevent acidification of cytoplasm G-6-P is preferably channeled into the central metabolic pathway as compared to pentose phosphate pathway. This preferential channeling may in turn increase the activities of TCA cycle dehydrogenases which may lead to an increased expulsion of protons entering the cytoplasm. In the case of *E. coli* W3110 phosphoacetyltransferase of the acetate production pathway is absent the acetyl-CoA, formed from G-6-P, is now solely entering into the TCA cycle leading to further increase in activities of dehydrogenase. The mechanism behind expulsion of protons by the studied dehydrogenases involves a recycling of reducing power between dehydrogenases of TCA cycle and ETC and, during this recycling excess of protons are pumped out of the cell. PTS is phosphoenolpyruvate-dependent phosphotransferase system; G6PD is glucose-6-phosphate dehydrogenase; ICD is isocitrate dehydrogenase; SDH is succinate dehydrogenase; MDH is malate dehydrogenase.

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APPENDICES

A truly happy person is one who can enjoy the scenery on a detour.

Appendix I

1. **LB Medium** (Luria Broth)**:**

   Composition per liter:

   Pancreatic digest of casein.....10.0 g

   NaCl.....5.0 g

   Yeast extract.....5.0 g

   pH 7.0 ± 0.2 at 25°C

   Preparation of Medium: Add components to distilled/deionized water and bring volume to 1.0 L. Mix thoroughly. Adjust pH to 7.0. Distribute into tubes or flasks. Autoclave for 25 min at 15 psi pressure –121°C.

   Use: For the cultivation of *Escherichia coli*.

2. **M9 Medium:**

   Composition per liter:

   Na_2HPO_4 .....6.0 g

   KH_2PO_4.....3.0 g

   NH_4Cl .....1.0 g

   NaCl.....0.5 g

   Glucose solution .....10.0 mL

   $MgSO_4 \cdot 7H_2O$ solution .....1.0 mL

   Thiamine·HCl solution.....1.0 mL

   $CaCl_2$ solution.....1.0 mL

   pH 7.0 ± 0.2 at 25°C

i. **Glucose Solution:**

   Composition per 100.0 mL:

   D-Glucose .....20.0 g

   Preparation of Glucose Solution: Add glucose to distilled/deionized water and bring volume to 1.0 L. Mix thoroughly. Autoclave for 15 min at 15 psi pressure –121°C.

ii. **$MgSO_4 \cdot 7H_2O$ Solution:**

   Composition per liter:

   $MgSO_4 \cdot 7H_2O$.....246.5 g

Preparation of $MgSO_4 \cdot 7H_2O$ Solution: Add $MgSO_4 \cdot 7H_2O$ to distilled/deionized water and bring volume to 1.0 L. Mix thoroughly. Autoclave for 15 min at 15 psi pressure – 121°C.

iii. Thiamine·HCl Solution:

Composition per 10.0 mL:

Thiamine·HCl .....10.0 mg

Preparation of Thiamine·HCl Solution: Add thiamine·HCl to distilled/deionized water and bring volume to 1.0 L. Mix thoroughly. Filter sterilize.

iv. $CaCl_2$ Solution:

Composition per liter:

$CaCl_2$ .....14.7 g

Preparation of $CaCl_2$ Solution: Add $CaCl_2$ solution to distilled/deionized water and bring volume to 1.0 L. Mix thoroughly. Autoclave for 15 min at 15 psi pressure –121°C.

Preparation of Medium: Add components, except $MgSO_4 \cdot 7H_2O$ solution, glucose solution, thiamine-HCl solution, and $CaCl_2$ solution, to distilled/ deionized water and bring volume to 987.0 mL. Mix thoroughly. Adjust pH to 7.0. Autoclave for 15 min at 15 psi pressure –121°C. Cool to room temperature. Aseptically add sterile $MgSO_4 \cdot 7H_2O$ solution, sterile glucose solution, sterile thiamine-HCl solution, and sterile $CaCl_2$ solution. Mix thoroughly. Distribute into tubes or flasks.

Use: For the cultivation and maintenance of *Escherichia coli* and a variety of other bacteria.

3. Nutrient Agar:

Composition per liter:

Agar .....15.0 g

Peptone.....5.0 g

NaCl.....5.0 g

Yeast extract.....2.0 g

Beef extract .....1.0 g

pH 7.4 ± 0.2 at 25°C

Source: This medium is available as a premixed powder from HiMedia.

Preparation of Medium: Add components to distilled/deionized water and bring volume to 1.0 L. Mix thoroughly. Gently heat and bring to boiling. Distribute into tubes or flasks. Autoclave for 15 min at 15 psi pressure –121°C. Pour into sterile Petri dishes or leave in tubes.

Use: For the cultivation and maintenance of a wide variety of microorganisms.

Appendix II

1. **Assay buffer:**

   pH 8.5 (0.2 M Tris, 1 mM $MgCl_2$)

   Composition per 1.0 L:

   Tris HCl…….7.23 g

   Tris base…….18.6 g

   $MgCl_2$…….203 mg

2. **BSA stock solution:**

   Make 10 mg/ml solution by adding 0.1 g BSA to the 10.0 mL distilled/deionized water.

3. **Glucose-6-phosphate solution:**

   100mM glucose 6 - phosphate solution prepared by addition of D-glucose 6- phosphate, monopotassium salt in 2 ml deionized water.

4. **Isocitrate Stock solution:**

   Add 11mg DL-isocitrate to the 10 mL assay buffer.

5. **$MgCl_2$ stock solution:**

   Make 0.1M solution by adding 20.33g $MgCl_2$ to the 1.0 L distilled/deionized water.

6. **$MnCl_2$ solution:**

   Make 0.1M solution by adding 19.8 g $MgCl_2$ to the 1.0 L distilled/deionized water.

7. **Monensin solution:** 1μM solution is prepared in 95% ethanol solution**.**

8. **NADH solution:**

   Make the solution by adding 0.567 g in 10 mL distilled/deionized water.

9. **$NADP^+$ stock solution:**

   Add 11mg of the $NADP^+$ to the 5 mL of assay buffer.

10. **Oxaloacetate solution:**

    Add 0.66 g oxaloacetate to the 10 mL assay buffer.

11. **Phosphate buffer:**

    Composition per 1.0 L:

    Na_2HPO_4…….1.4 g

    KH_2PO_4 ……0.02 g

    NaCl……2.0 g

12. **Sodium benzoate solution:** 20mM solution is prepared in distilled/deionized water.

13. **Tris HCl buffer:**

    pH 7.5 (Stock Solution)

    0.2 M Tris Base…….…… 24.2 g in one liter

    0.2 M HCl …….16.7 ml of 12 N HCl in one liter

    1.0 mM $MgCl_2$..... 2.03 g in 10 mL