Optimized Conditions for Increasing *Escherichia coli* Resistance to p-Hydroxybenzoic Acid

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Abstract: The present study aimed at increasing resistance of *Escherichia coli* to p-hydroxybenzoic acid (pHBA) through manipulation of different environmental and physiological factors. According to the study, different incubation temperatures, pHs, agitation rates and medium components were tested to characterize *E. coli* resistance to pHBA in shake flask and a laboratory fermentor. Genetic analysis using PCR of four representative *E. coli* isolates showed that *yhcP* gene was detected in both sensitive and resistant wild isolates of natural sources, a finding that stressed the importance of studying different environmental, physiological and genetic factors affecting the regulation of *yhcP* gene. MIC of pHBA against *E. coli* strain BW25113 that has the YhcP efflux pump showed a 64 fold increase by changing the growth medium from nutrient broth to basal medium containing 2% peptone and 2.6% glucose and keeping the pH constant at 8. Increased resistance of *E. coli* to pHBA could provide an effective solution to the toxicity of acid to the producing host bacterial cell which in turn will help to increase production of this molecule for commercial use. [Journal of American Science 2010;6(7):155-169]. (ISSN: 1545-1003).

Keyword: *Escherichia coli*, p-hydroxybenzoic acid, environmental, physiological factors, *yhcP* gene

1. Introduction

E. coli resistance to pHBA is mediated through an efflux pump (Van Dyk *et al*., 2004). Efflux systems export toxic metabolites from the cells, and consequently are capable of conferring resistance to different chemotherapeutic agents. Transport proteins taking part in the extrusion of noxious agents may be specific for one or for a range of structurally different substrates (Kern et al., 2000). Five families of bacterial efflux systems have been identified (Putman *et al*., 2000). These include: i) the small multidrug resistance family SMR (a member of the much larger drug⁄metabolite transporter (DMT) superfamily (Poole, 2004); ii) the resistance nodulation division (RND) family (Zgurskaya and Nikaido, 2000), which is part of the larger RND permease superfamily (Tseng et al., 1999); iii) the major facilitator superfamily MF (Pao et al., 1998); iv) the ATP binding cassette family (ABC) (van Veen and Konings, 1998); and v) the multidrug and toxic compound extrusion family MATE (Brown *et al*.,1999).

Treatment of *E. coli* with pHBA resulted in upregulation of *yhcP*, encoding a protein of the putative efflux protein family & the adjacent genes *yhcQ*, encoding a protein of the membrane fusion protein family, and *yhcR*, encoding a small protein without a known or suggested function. The function of the upstream, divergently transcribed gene *yhcS*, encoding a regulatory protein of the LysR family, in regulating expression of *yhcRQP* was shown. The efflux function encoded by *yhcP* was proven by the hypersensitivity to pHBA of a *yhcP* mutant strain. A *yhcS* mutant strain was also hypersensitive to pHBA. Expression of *yhcQ* and *yhcP* was necessary and sufficient for suppression of the pHBA hypersensitivity of the *yhcS* mutant. Only a few aromatic carboxylic acids of hundreds of diverse compounds tested were defined as substrates of the YhcQP efflux pump. Thus, a proposal for renaming yhcS, yhcR, yhcQ, and yhcP, was made to reflect their role in aromatic carboxylic acid efflux, to aaeR, aaeX, aaeA, and aaeB, respectively. The role of pHBA in normal *E. coli* metabolism and the highly regulated expression of the AaeAB efflux system suggest that the physiological role may be as a "metabolic relief valve" to alleviate toxic effects of imbalanced metabolism (Van Dyk et al., 2004).

pHBA is a key monomer used in the production of liquid crystalline polymers (LCPs) and its cost is a major portion of the high cost of LCPs, and limits the commercial applications for which they can be used. A large proportion of the cost of pHBA, and consequently LCPs, is related to the cost of its chemical synthesis (Amaratunga *et al*., 2000). pHBA has an additional utility as a chemical intermediate for the manufacture of paraben preservatives and other products (Johnson *et al*., 2000).

Earlier reports (Amaratunga *et al*., 2000); (Johnson *et al*., 2000) of improved biosynthesis of pHBA relied on plasmid localization of *ubiC* to achieve significant overexpression of chorismate lyase. The use of ion-exchange resin to remove product during fermentation runs had a particularly pronounced impact in the earlier report (Johnson *et al*., 2000) on microbial production of pHBA. In the absence of product removal using ion-exchange resin during the course of a fermentation run, 6.2 g/L of pHBA was synthesized by the construct (Amaratunga *et al*., 2000)

that resulted from two rounds of selection for resistance to antimetabolites and over-expression of plasmid-localized *ubiC*. However, a total of 22.9 g/L of pHBA was synthesized when anion exchange resin was used to remove product during the course of the fermentation (Johnson *et al*., 2000).

By repeatedly passing culture medium through the anion-exchange resin during the fermentation, the concentration of pHBA in the culture medium was likely kept at a level that reduced its toxicity towards the producing microbe. This is not to suggest that pHBA toxicity was completely avoided. It was observed that a substantial reduction in cell biomass and product concentration at concentrations of pHBA of 10 g/L. More effective solutions to the toxicity of pHBA have to be found given that pHBA negatively impacts *E. coli* metabolism even at 10 g/L concentrations under fed-batch fermentor conditions. Several methods of producing aromatic carboxylic acids from recombinant microorganisms are described in the literature, however it will be advantageous to optimize production of these molecules for commercial use, route to optimized production is increased yield, as many of these aromatic carboxylic acids are toxic to the producing host cell, another route may be to minimize the toxic effect the end product has on the host cell. A family of ubiquitous the proteins that may be able to address both of these issues are the efflux proteins (Sariaslani and Van Dyk, 2007).

This proposed study aimed at optimizing a simple fermentation method for increasing resistance of *E. coli* BW25113 which has *yhcP* gene that codes the efflux pump of pHBA. The study was conducted on flasks level and in a laboratory fermentor.

2. Materials and methods

Microorganisms

E. coli standard strains*: E. coli* BW25113 which has *yhcP* gene that codes the efflux pump of pHBA & *E. coli* BW25113Δ*yhcP* which lacks such gene.

E. coli isolates: a total number of 38 isolates were isolated from different sources and identified biochemically according to Bartelt,1999 then categorized according to the isolation source into clinical isolates coded (1,2,3,9,10,11,17, 18, 19, 23, 24, 25, 26, 27, 28, 29, 20, 21, 22, 30) & isolates from food handlers coded (12, 13, 14, 15, 31, 32, 33, 34, 35, 36, 37, 38) & isolates from veterinary source coded (39, 40) & isolates from miscellaneous sources (4, 5, 6, 16).

Media and chemicals

All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Merck (Germany), Biolife (Italy), Britania (Argentine), Oxoid (England), or Difco laboratories (Detroit, USA). The basal medium used is composed of: Ammonium sulphate (0.500 gm), Calcium chloride.$2H_2O$ (0.001 gm), Magnesium sulphate.$7H_2O$ (0.05gm), Di-Potassium hyderogen orthophosphate (1.740gm), Potassiom dihydrogen orthophosorthophosphate (1.360 gm), distilled H_2O to 100 ml, peptone or beef extract were used as protein source & glucose was incorporated to the required concentration by adding a specified volume from sterilized glucose stock solution after autoclaving.

Determination of susceptibility of *E. coli* isolates/strains to pHBA

The susceptibility of the tested *E. coli* isolates/strains to pHBA was assessed by determining the MIC against it. The MIC was determined by microdilution technique according to Van Dyk et al., 2004 with some modifications. The inoculum was 10 µL prepared by suspending a fresh slant of the tested *E. coli* isolate/strain in 3 ml 0.9% physiological saline solution. The turbidity of the suspension was adjusted to 0.5 McFarland standards ($1\text{-}2x10^8$ cfu/ml). The suspension was further diluted one thousand fold to yield $1\text{-}2x10^5$ cfu/ml. The plates were incubated at 37^oC for 24 hours. The MIC of the resulting diluted suspension was determined in nutrient broth against pHBA. For each tested isolates/strains, duplicate measurements were conducted.

Studying the different environmental factors affecting the susceptibility of some *E. coli* isolates/strains to pHBA in shake flasks

The effect of different environmental factors on susceptibility of the two standard *E. coli* strains (BW25113 and BW25113Δ*yhcP*) and eight selected *E. coli* isolates to pHBA was studied. Five of the ten tested isolates/strains were of high MIC values (7, 9, 15, 20, and 37) and the other five were of low MIC values (3, 8, 24, 25 and 34). The different factors studied included; agitation rate, incubation temperature and initial pH. The experiment was carried out in 100 ml Erlenmeyer flasks, each containing 25 ml nutrient broth. The flasks were inoculated with the bacterial suspension (10^5 cfu/ml) of the test strain at 2% v/v inoculum size. The inoculum of the test strain was prepared as described in the determination of susceptibility of *E. coli* isolates/strains to pHBA. The stock solution of pHBA (500 mM) was incorporated in the culture flasks to the final concentration of 2.5 mM. Control flasks were similarly prepared but contained no pHBA. The flasks were incubated in an incubator shaker (ROMO Company, local supply) at the specified temperature and agitation rate. In case of studying the effect of initial pH only, the initial pH of the culture medium was adjusted before autoclaving to the required value. In all cases, aliquots were removed at different time intervals for determination of O. D at 600 nm.

Effect of different culture medium components on the MIC of pHBA against the tested *E. coli* isolates/strains

The MIC values of pHBA against the two standard *E. coli* strains (BW25113 and BW25113Δ*yhcP*) and the eight selected *E. coli* isolates to pHBA were determined in microtitre plate principally as described in the determination of susceptibility of *E. coli* isolates/strains to pHBA. The inoculum was prepared in shake flasks using a culture medium similar to the corresponding medium used for MIC determination in each case. The flasks (100 ml) contained 25 ml culture medium were inoculated with 0.5 ml aliquots of 10^8 cfu/ml of fresh bacterial suspension prepared as described in the determination of susceptibility of *E. coli* isolates/strains to pHBA and incubated overnight at 37°C and 320 rpm.

The optical density of the growth obtained was determined and the count was then adjusted to10^8 cfu/ml using the equation 1 O. D_{600} = $1x10^9$ cfu/ml. The 1 O. D_{600} = 10^9 cfu/ml /ml expressed the average value of 1 O. D_{600} = $8x10^8$ cfu/ml (Becker *et al*., 1996) and 1 O. D_{600} = $1.7x10^9$ cfu/ml (determined experimentally), and then serially diluted with physiological saline to 10^5 cfu/ml. The adjusted bacterial suspension obtained was used as inoculums for MIC determination in microtitre plate. The different factors studied included variable concentrations of sodium and potassium chloride salts (0.5, 1 and 2%), tween 80 (1 and 2%), 1.5% (glucose and sucrose) and 1% starch, and different protein/glucose combinations. All factors were studied in nutrient broth except the different protein/glucose combinations the used protein source was added to the basal medium (described in materials and methods) containing either 2.6% or 5 % glucose. The protein source was added at 2% final concentration. In addition the two protein sources (beef extract and peptone) were separately tested at 4% final concentration in basal medium containing only 2.6% glucose.

Genetic analysis of some selected *E. coli* isolates for their acquisition of the pHBA efflux pump gene, *yhcP*

Four isolates were subjected to PCR amplification for screening of the *yhcP* gene as two isolates of high MIC values (isolates 9 and 37) and the other two were of low MIC values (isolates 3 and 34) against pHBA. *E. coli* strains BW25113 (strain 7) and BW25113Δ*yhcP* (strain 8) were used as positive and negative controls, respectively.

The extraction of the chromosomal DNA was carried out according to Wu, *et al*., 2004. The DNA primers used in the PCR reaction were: Left primer: GCGTCAGCGATAGCGTATTG and right primer: GTGGATCGAGAGCTGGAAAG.

The PCR reaction mixture: Buffer (2.5 ml), $MgCl_2$ (1µL), (20 pico) primer R (2 µL), (20 pico) primer left (2 µL), (10 mM) dNTP's (0.5 µL), template DNA (2 µL), Taq (0.25 µL), PCR H_2O (14.75 µL). Total volume (25 µL).

The PCR reaction conditions were: initial denaturation at 94°C for 3 minutes and then 40 cycles, each consisted of (1) denaturation at 94°C for 1 minute, (2) annealing at 56°C for 1 minute, (3) extension at 72°C for 1 minute. A post-extension period at 72°C for 7 minutes was allowed. The PCR product was approximately 200 bP.

Fermentor experiments

Fermentation process. Vessel preparation with culture medium

The culture medium applied was basal medium containing 2% peptone to which glucose was added after sterilization to the final concentration of 2.6%. Sterile glucose solution was incorporated from 5% glucose solution (500 ml plastic bottles) which was obtained from a local commercial source and their labeled concentration was checked experimentally using glucose quantization kits. The culture medium was dispensed in BioFlo 110 advanced fermentation kit equipped with a heat blanketed 5 L glass fermentation vessel and the different fermentation processes were carried out using 3 liters working volume. The vessel containing the culture medium and the liquid addition bottles of acid, base and antifoam were sterilized batch wise in an autoclave at 121°C for 15 minutes. Aeration was conducted by admission of compressed air after its passage through two types of filters, the first filter was located in the front of the air exit from the compressor and it was used for the removal of dust and graze while the second one (resterilizable cartridge bacterial filter, 0.2 µm) was located just above the vessel head plate and was connected to the sparger by an autoclavable silicon tube.

The cartridge filter was sterilized by autoclaving while connected to the vessel. The aeration was controlled at 3 vvm for the different fermentation cycles. The dissolved oxygen was monitored during the fermentation cycles by the aid of dissolved oxygen probe which was calibrated after sterilization and before inoculation to the scale zero and 100. The readings were recorded during the fermentation cycles manually. Temperature was detected by a thermowell and controlled automatically through the fermentor microprocessor at 37°C by the use of heat blanket and internal cooling coils installed inside the vessel. The agitation was conducted by a motor (Magmotor Corporation a

SatCon Co.) connected to the agitator shaft above the vessel head plate and operated at a fixed rate of 200 rpm for the different fermentation cycles. The pH was controlled automatically to the tested values (6, 7, 8) using 5 M NaOH and 1 N HCL and by the aid of sterilizable pH probe which was calibrated before use. NaOH and HCl bottles were connected to the acid and base ports by autoclavable silicon rubber tubes passed through peristaltic pumps. Foaming was monitored visually and suppressed by the addition of soybean oil (Agriculture research center, soybean research institute) using the manual mode of the primary control unit.

Inoculum build up

The inoculum was prepared in shake flasks using a culture medium similar to that used for cell growth in the fermentor. The flasks (250 ml) contained 60 ml culture medium were seeded with 2% V/V of 10^8 cfu/ml fresh bacterial suspension prepared as described in the determination of susceptibility of *E. coli* isolates/strains to pHBA and incubated overnight at 37^{o}C and 320 rpm. The inoculum was added to the vessel manually through the inoculation port at an inoculums size of 2% V/V after establishment of different operating parameters (temperature, pH and dissolved oxygen).

Effect of pH on growth profile and susceptibility to pHBA of the tested *E. coli* strains grown in the fermentor

A number of fermentation cycles for the two *E. coli* strains BW25113 and BW25113Δ*yhc*P were carried out at constant pH values of (6, 7 and 8) in laboratory fermentor (New Brunswick Scientific's BioFlo® 110 fermentor).

At the different tested pHs, samples were withdrawn at the time intervals of 0, 2, 4, 6, 8, 10, and 11 hour and at the end of the fermentation cycle (about 24 hours), appropriately diluted with physiological saline and their optical densities were determined with spectrophotometer at 600 nm to obtain the growth profile.

The growth obtained was tested for susceptibility to pHBA using two approaches (MIC determination and Killing Kinetics); both were carried out on a sample collected after 6 hours growth in the fermentor. The stock pHBA solutions used for MIC determination and killing kinetics studies were prepared at the tested pHs of 6, 7 and 8.

1- Determination of MIC using cells grown in the fermentor as inocula

MIC was determined in microtitre plates using a culture medium similar to that used for cell growth in the fermentor and of the same pH (6, 7 or 8). The pH of the medium used for MIC determination in the microtitre plate was adjusted before autoclaving then rechecked and corrected to the required pH after sterilization using sterile NaOH (4M) or HCl (1M).The cells collected after 6 hours growth in the fermentor were harvested by centrifugation (6000 rpm for 5 minutes) in a cooling centrifuge. Then, the cell pellets were washed twice with physiological saline then resuspended in physiological saline with the same pH of the fermentor medium used in each run (6, 7 or 8). The resulting bacterial suspension was adjusted to a viable count of 1-2x10^8 cfu/ml using 0.5 McFarland standard then serially diluted with physiological saline of the same pH to 10^5 cfu/ml. The adjusted bacterial suspension obtained was used as inoculums for MIC determination in the microtitre plate.

Stock solution of pHBA adjusted to the same pH of the growth medium was serially diluted in the wells of the microtitre plate. The wells of the microtitre plates were inoculated by 10 µL aliquots of the bacterial suspension under test. The plates were incubated at 37^{o}C for 24 hours and the MICs were determined by visually assessing turbidity after 24 hours. For each tested strain, triplet measurements were conducted.

2- Killing Kinetics determination

The bacterial cell suspension was prepared as described above then standardized by viable count. The killing kinetics experiment was carried out using stock pHBA solution of the same pH of the fermentor medium used in each run (6, 7 or 8). The bacterial suspension (2 ml) was mixed with (20, 251, 666µL) of the stock pHBA solution (2048, 1430, 1280 mM) in a Wassermann tube to give (20, 160, 320 mM) final concentration respectively. Aliquots (100 µL) were withdrawn at different time intervals (0, 2,5,50 minutes) for pH 6 and (0, 18, 103, 230 minutes) for pH 7 and 8. Aliquots were serially diluted in test tubes containing 10 ml physiological saline solution of the same pH. Then, the count of the residual surviving cells was determined by surface viable count by spreading method (Brown *et al*., 1989) using nutrient agar as growth medium. Then, a death curve between the number of survivors and time intervals was constructed.

3. Results

Susceptibility of *E. coli* isolates and other *E. coli* reference strains to pHBA

The MIC values of pHBA against the collected *E. coli* isolates/strains were determined by microdilution technique in microtitre plates and the results are shown in Table 1.

Environmental factors affecting the susceptibility of some *E. coli* isolates/strains to pHBA in shake flasks

The effect of agitation rate

It was studied by varying the RPM (0, 140, 240 and 320) of the incubator shaker. The results displayed in Figures 1 & 2 as representatives of the ten isolates showed that the isolates of the code numbers 3 & 8 didn't exhibit growth in the presence of pHBA in comparison to control isolates (without pHBA). The same happened with isolates 24, 25 and 34 (data not shown).

However, the isolates coded 7 & 9 showed an increase in growth in the presence of pHBA by increasing the rpm in most cases. The same happened with isolates 15, 20 and 37 (data not shown). For most isolates their growth was comparable to that of the controls (without pHBA). The agitation rate of 320 rpm showed maximum growth with most isolates and it was applied in the subsequent experiments conducted in shake flasks.

Effect of incubation temperature

It was studied at two temperature degrees (37^{o}C and 40^{o}C) and at 320 rpm in shake flasks. The isolates having the code numbers of 3 & 8 showed no appreciable growth in the presence of pHBA for the two tested temperature degrees in comparison to control isolates (without pHBA) Figures 3 &4. The same happened with isolates 24, 25 and 34 (data not shown). However, for isolates of code numbers 7 & 9 the results showed that their growth in the presence of pHBA increased at both the tested temperature degrees (in most cases) becoming nearly equal to these of controls (without pHBA). The same happened with isolates 15, 20 and 37 (data not shown).

Effect of initial pH

Before studying the effect of initial pH on susceptibility of the ten selected *E. coli* isolates to pHBA, both growth and pH profiles of the tested isolates/strains were monitored for cells grown in nutrient broth and at different initial pH values. The results are shown in Figures 5 & 6. Both *E. coli* strains 7 and 8 behaved alike at the different tested initial pHs regarding growth and pH profiles. The two isolates 3 and 9 also behaved alike regarding pH profile although they showed different growth values at some data points. The resistant isolate 9 showed higher growth after 5 hours of incubation than the sensitive isolate 3 at the different tested pHs. Both isolates 3 and 9 showed similar maximum growth values at the different tested pHs. The susceptibility of the selected isolates/strains to 2.5 mM pHBA at three different initial pH values (5.3, 5.7 and 6.8) was studied. At the initial pH 5.3 both sensitive and resistant isolates/strains showed no growth at all data points. For the sensitive isolates, the effect of pHBA was aggravated at the initial pH 5.7 where nearly no growth was observed with the different isolates (Figures 7 & 8). In contrast, the resistant isolates showed high growth values except for the strain 7. At the initial pH 6.8, still high growth was obtained with the resistant isolates compared to the corresponding sensitive isolates in most cases. The pH profiles for resistant isolates showed differences compared to those of the sensitive isolates at some data points.

Effect of different culture medium components on the MIC of pHBA against the tested *E. coli* isolates/strains:

Effect of variable concentrations of sodium and potassium chloride salts

Results in Figure 9 showed that for isolates 8, 3, 24, 25 and 34 which have low MIC values for pHBA, their MIC increased from 2.5 mM (Table 1) to 5 mM (became equal to those of isolates 7, 9, 15, 20 and 37) with the addition of sodium chloride to nutrient broth at all concentrations tested (0.5, 1 and 2%). The same pattern was obtained with the addition of potassium chloride to nutrient broth at concentrations of 0.5 and 2% but not at 1% where the isolate 34 showed even higher increase in MIC to be equal to 10 mM. However, for isolates 7, 9, 15, 20 and 37 which have high MIC values for pHBA, the results displayed in the same Figure showed that the susceptibility of the isolates to pHBA was not affected by addition of any concentration (0.5, 1, 2%) of KCl or NaCl to nutrient broth except for the isolate 37, the MIC increased to 10 mM at 1% KCl concentration, compared to 5 mM in absence of KCl.

Effect of variable concentrations of tween 80

Results in Figure 10 showed that for isolates which have low MIC values for pHBA (Table 1), their MIC values increased from 2.5 mM to 5 mM with the addition of tween 80 to nutrient broth at both concentrations tested (1 and 2%) while isolate 34 showing even higher increase in MIC to be 10 mM with 2% tween 80. However, for isolates 7, 9, 15, 20 and 37 which have high MIC values for pHBA (Table 1), the results displayed in the same Figure showed that the susceptibility of these isolates to pHBA was not affected by addition of tween 80 to nutrient broth at concentration of 1% where their MIC values remained unchanged (5 Mm). While the addition of 2% tween 80 to nutrient broth increased the MIC values of isolates 7, 9, and 37 to be 10 mM.

Effect of some carbohydrate sources

For isolates 8, 3, 24, 25 and 34, the following results were displayed in Figure 11: the

Table 1. Summarization of *E. coli* isolates/strains from various sources according to their MIC values against pHBA

	MIC (mM)	Number of isolates/strains (their code numbers) from various sources according to their MIC values				
		Reference strains	Clinical isolates	Isolates from food handlers	Isolates from veterinary source	Miscellaneous isolates
pHBA	5	2 (BW25113-16)	13 (1,2,9-11, 17-23, 29)	5 (12-15, 37)	-	2 (4,6)
	2.5	1 (BW25113Δ*yhcP*)	7 (3, 24-28, 30)	7 (31-36, 38)	2 (39, 40)	1 (5)

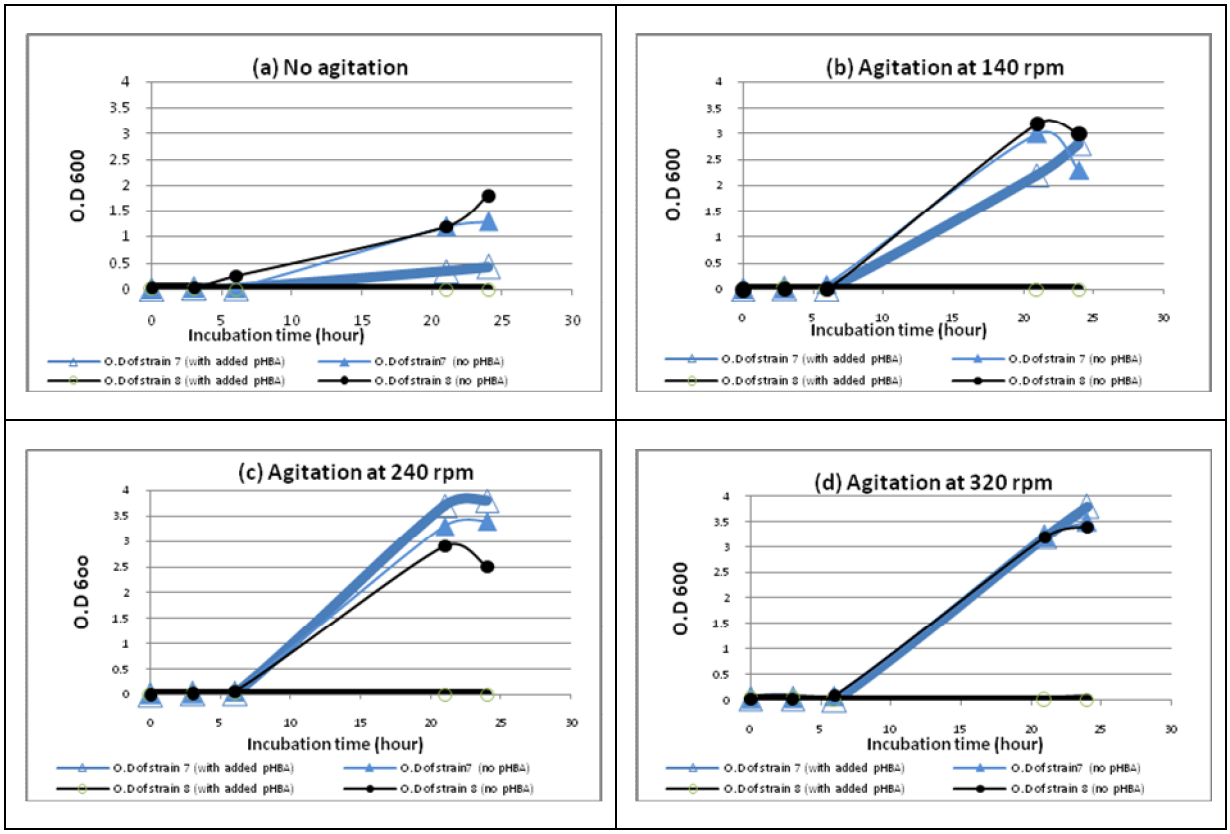
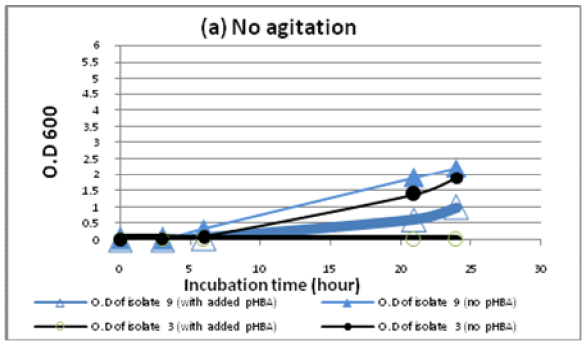
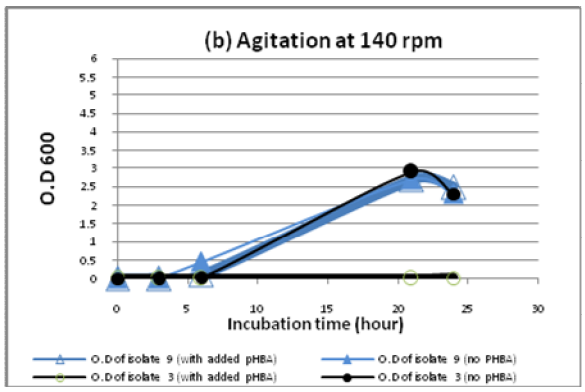
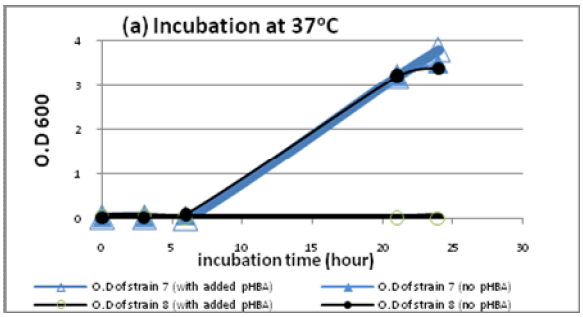


Figure 1. Effect of agitation rate on susceptibility of *E. coli* strains BW25113 (strain 7) & BW25113Δ*yhcP* (strain 8) to 2.5 mM pHBA in shake flasks







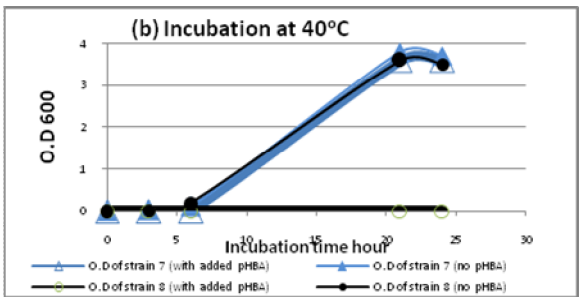
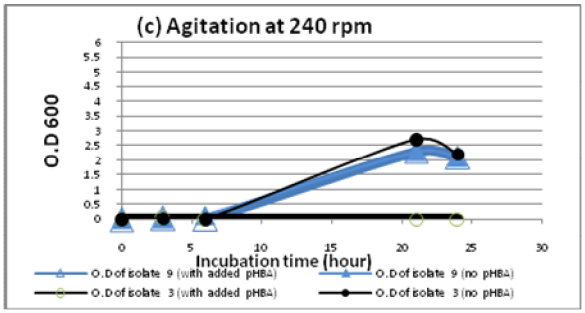


Figure 3. Effect of incubation temperature on susceptibility of *E. coli* strains BW25113 (strain 7) and BW25113Δ*yhcP* (strain 8) to 2.5 mM pHBA in shake flasks.



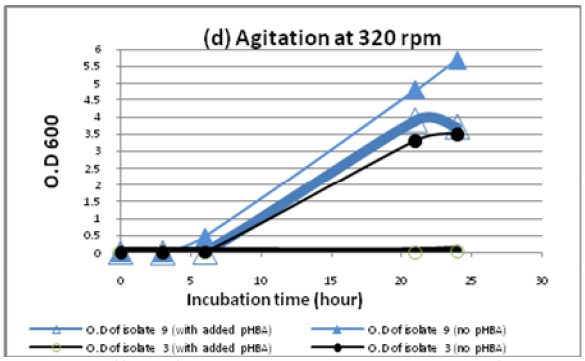
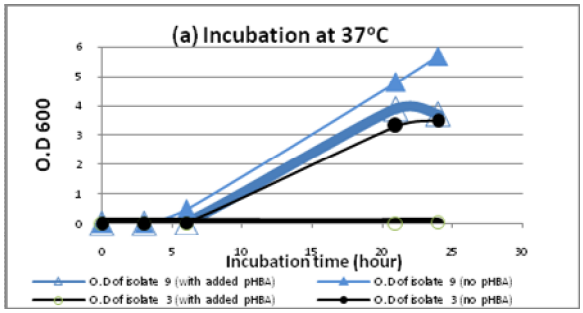


Figure 2. Effect of agitation rate on susceptibility of *E. coli* isolates 9 and 3 to 2.5 mM pHBA in shake flasks



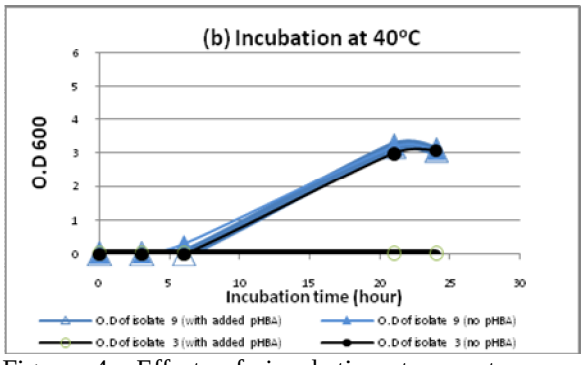


Figure 4. Effect of incubation temperature on susceptibility of *E. coli* isolates 9 and 3 to 2.5 mM pHBA in shake flasks

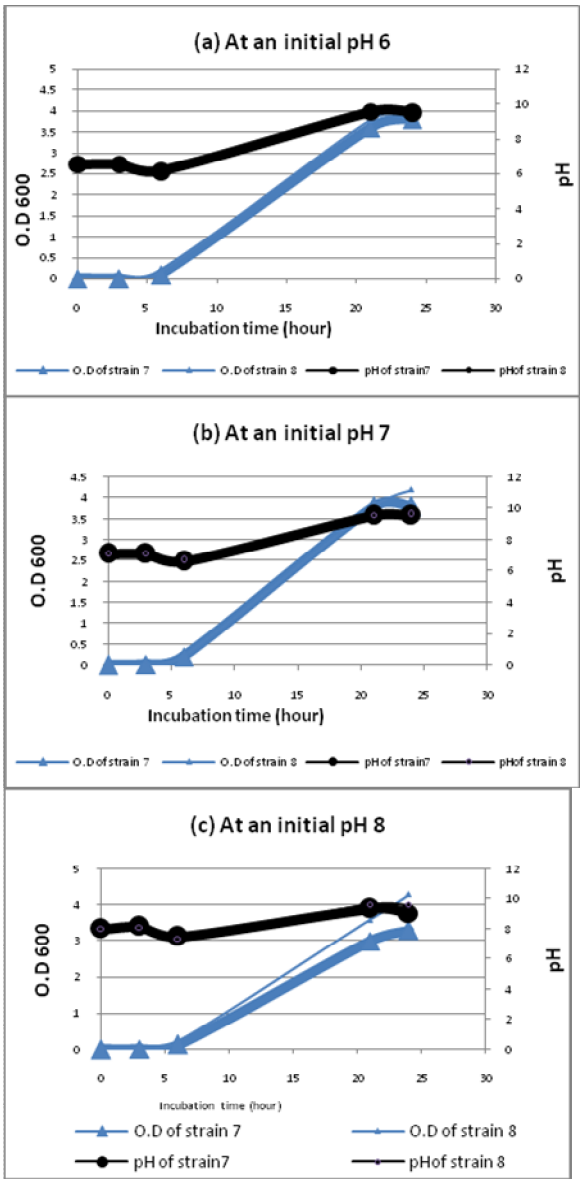


Figure 5. Growth and pH profiles of *E. coli* strains BW25113 (strain 7) and BW25113Δ*yhcp* (strain 8) grown in nutrient broth at initial pHs of 6, 7 and 8 in absence of pHBA

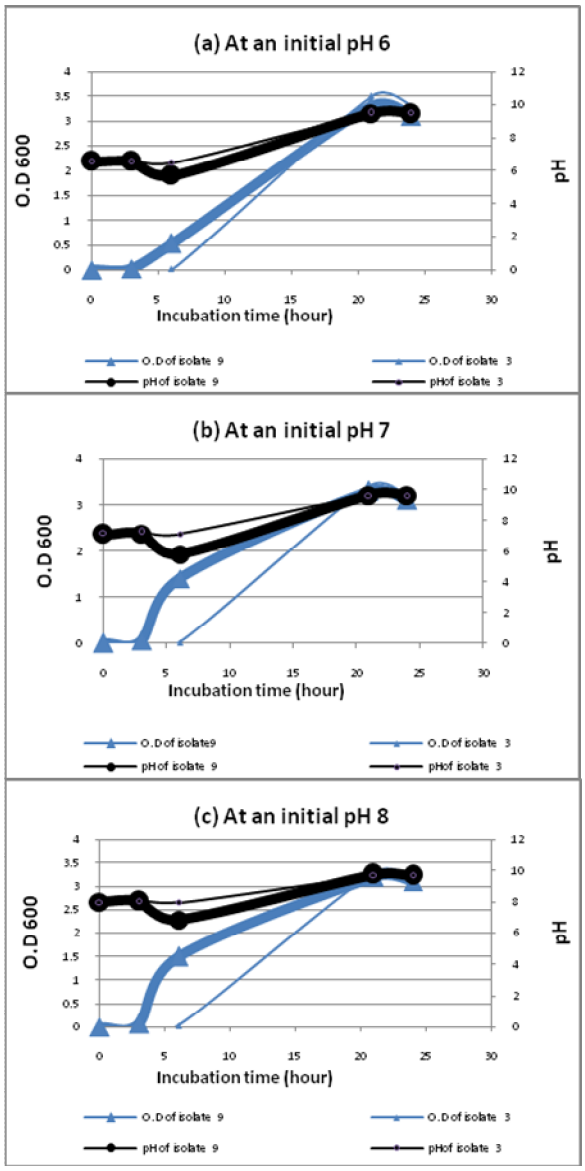
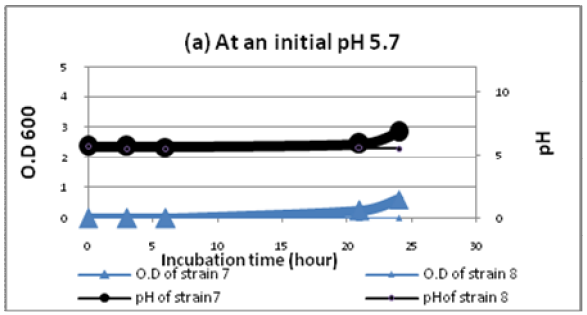


Figure 6. Growth and pH profiles of *E. coli* isolates 9 and 3 grown in nutrient broth at initial pHs of 6, 7 and 8 in absence of pHBA



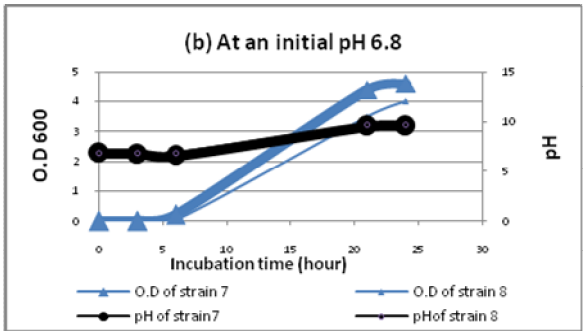
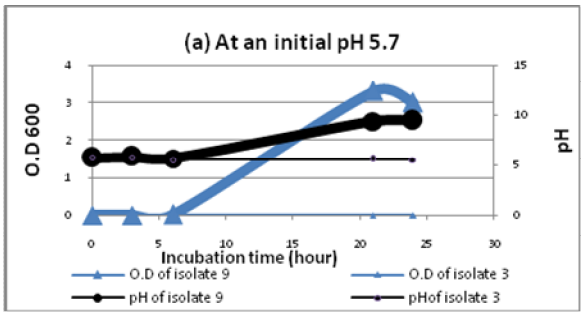


Figure 7. Growth and pH profiles of *E. coli* strains 7 and 8 grown in nutrient broth at initial pHs of 5.7 and 6.8 in presence of 2.5 mM pHBA



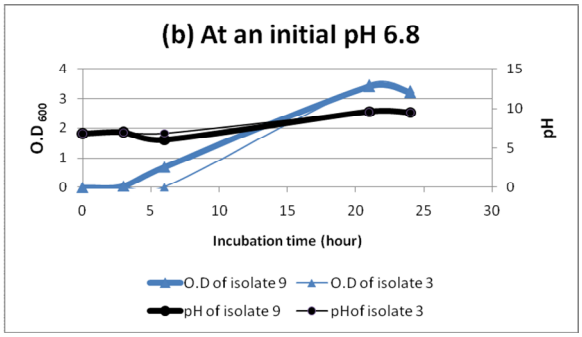


Figure 8. Growth and pH profiles of *E. coli* isolates 9 and 3 grown in nutrient broth at initial pHs of 5.7 and 6.8 in presence of 2.5 mM pHBA

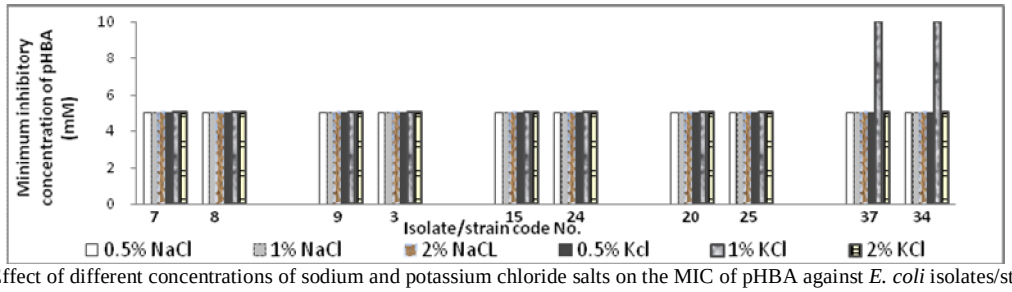


Figure 9. Effect of different concentrations of sodium and potassium chloride salts on the MIC of pHBA against *E. coli* isolates/strains as determined by microdilution technique. The tested agent was added to nutrient broth.

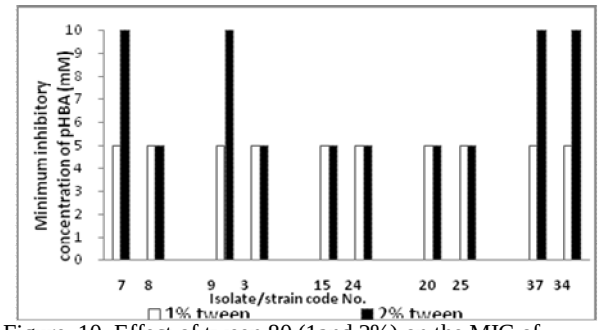


Figure 10. Effect of tween 80 (1and 2%) on the MIC of pHBA against *E. coli* isolates/strains as determined by microdilution technique. The tested agent was added to nutrient broth.

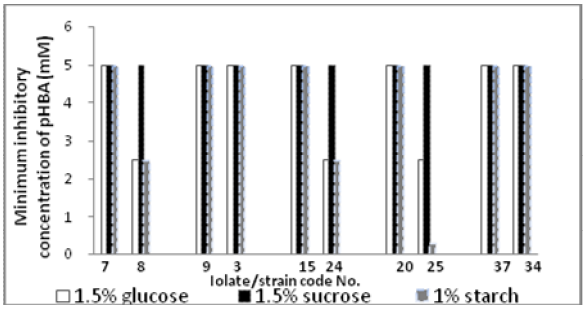


Figure 11. Effect of glucose (1.5%), sucrose (1.5%) and starch (1%) on the MIC of pHBA against *E. coli* isolates/strains as determined by microdilution technique. The tested agent was added to nutrient broth

addition of glucose (1.5%) caused no change in the MIC values (2.5 mM) of isolates 8, 24 and 25 while such addition caused two fold increase in the MIC values of isolates 3 and 34. The addition of sucrose (1.5%) to nutrient broth increased the MIC values from 2.5 mM to 5 mM for all these five isolates. The addition of starch (1%) increased the MIC values from 2.5 mM to 5 mM of isolates 3 and 34 while the MIC of isolate 25 sharply decreased to 0.3125 mM (8 fold reduction). However, for isolates 7, 9, 15, 20 and 37 the susceptibility to pHBA was not affected by addition of glucose (1.5%) , sucrose (1.5%) or starch (1%) to nutrient broth to (the MIC remained 5 mM).

Effect of different protein/glucose combinations

At peptone/glucose ratios of 2/2.6 and 2/5 and for the same test isolate no change in MIC values was observed except for isolate 9 where its MIC value decreased at high glucose concentration (Figure 12). At peptone/glucose ratios 2/2.6 and 4/2.6 and for the same test isolate, increasing protein concentration caused a decrease in MIC values of isolate 9, an increase in MIC values of isolates 24 and 37 and no change in MIC values of other isolates (Figure 12).

Comparison of the MIC values of the pHBA resistant isolates with those of their corresponding sensitive isolates, revealed that: the overall pattern showed a difference in MIC values with one condition or more for strain 7 versus strain 8 which reached 4 fold, isolate 9 versus 3, isolate 15 versus 24 and isolate 37 versus 34.

However, no such difference in MIC values was observed with isolate 20 versus 25 (Figure 12).

For beef extract/glucose combinations the following was observed: At beef extract/glucose ratios of 2/2.6 and 2/5 and for the same test isolate no change in MIC values was observed except for isolate 8 where high MIC value was obtained at high glucose concentration (Figure 13). At beef extract /glucose ratios 2/2.6 and 4/2.6 and for the same test isolate, increasing protein concentration caused no change in MIC values except for isolate 8 where high MIC value was obtained at high protein concentration (Figure 13).

Comparing the MIC values of the pHBA resistant isolates with those of their corresponding sensitive isolates, the following was observed: the overall pattern revealed a difference in MIC values for only strain 7 versus strain 8. While such difference in MIC values became nearly absent with other isolates (Figure 13).

PCR detection of the pHBA efflux pump gene, *yhcP* in four *E. coli* isolates

Results in Figure 14 showed a clear intensive band exhibited by each tested isolate and of size corresponds to that of the positive control.

Fermentor experiments

The cell growth and dissolved oxygen profiles of the two strains under the different tested pH values are shown in Figure 15. Both strains showed similar dissolved oxygen profiles at the different tested pHs (6, 7 and 8) while some differences between the two strains were noted in the growth profiles especially at pH 6 and 8. The results of killing kinetics for both strains are presented in Figure 16; they clearly exhibited different killing patterns. Almost in all cases, the killing kinetics rate of strain BW25113Δ*yhcP* was greater than that of strain BW25113 especially in the early periods of exposure to pHBA. The results of MIC determination of pHBA against the two tested strains at the different tested pH values are represented in Table 2.

4. Discussion

A better understanding of medium components and environmental factors affecting the susceptibility of some *E. coli* strains to pHBA on flasks level can be used to improve their resistance to pHBA.

The results displayed in Figures 1 & 2 showed that the susceptibilities of isolates of code numbers 3&8 to pHBA weren't affected at any rpm (140, 240, 320) or in absence of agitation.

However, the susceptibility to pHBA of the isolates coded 7& 9 was affected for agitated cultures in comparison to non agitated ones. In comparison to control, agitation supported growth in the presence of pHBA. Higher agitation rates (240 & 320 rpm) showed nearly similar growth for the tested isolate in the presence and absence of pHBA. In addition, in most cases higher agitation rate (320 rpm) produced more growth than lower agitation rates either in the presence or absence of pHBA. The agitation rate of 320 rpm was used by Amaratunga *et al*.,2000 in shake flask for increasing production of pHBA from *E. coli*. This may be attributed to the fact that stirring accomplishes two things: It mixes the gas bubbles through the liquid and it mixes the organism through the liquid, thus ensuring uniform access of microbial cells to the nutrients (Madigan *et al*., 2003).

The temperature in a vessel or pipe is one of the most important parameters to monitor and control in any process (Stanbury *et al*., 1995). The results displayed in Figures 3 & 4 showed that that the susceptibilities of isolates of code numbers 3&8 to pHBA weren't affected at any tested temperature in the presence of pHBA. However, the susceptibility to pHBA of the isolates coded 7& 9 was affected as they showed similar growth in the presence and in absence of pHBA in most cases at both the two tested temperature degrees (37, 40°C). However, in a number of cases the growth attained at 37◦C was higher than that attained at 40◦C. The temperature 37◦C is the incubation temperature used for pHBA MIC determination and

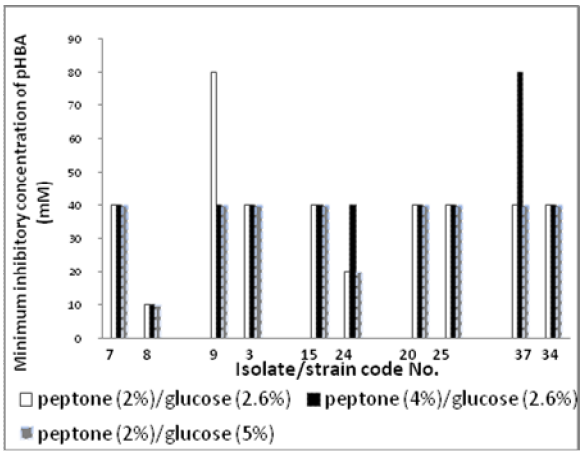


Figure 12. Effect of different peptone/glucose combinations on the MIC of pHBA against *E. coli* isolates/strains as determined by microdilution technique. Peptone/glucose combinations were added to the basal medium

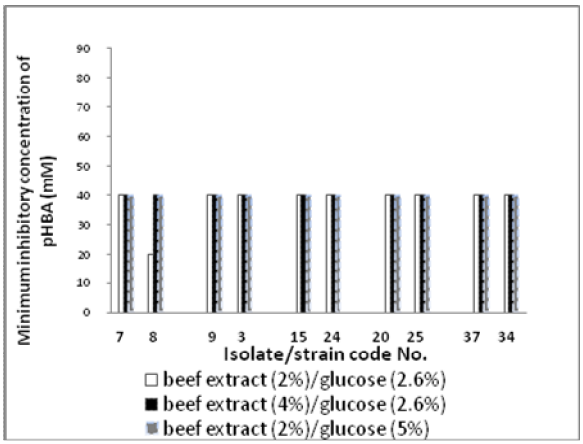


Figure 13. Effect of different beef extract/glucose combinations on the MIC of pHBA against *E. coli* isolates/strains as determined by microdilution technique. Beef extract/glucose combinations were added to the basal medium .

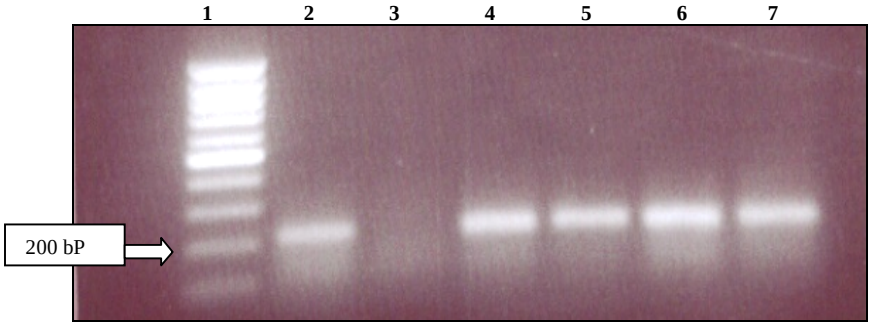
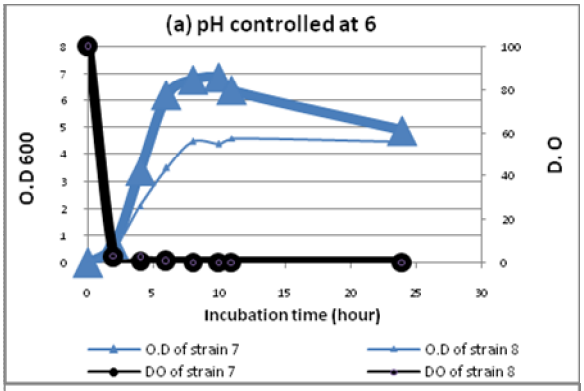


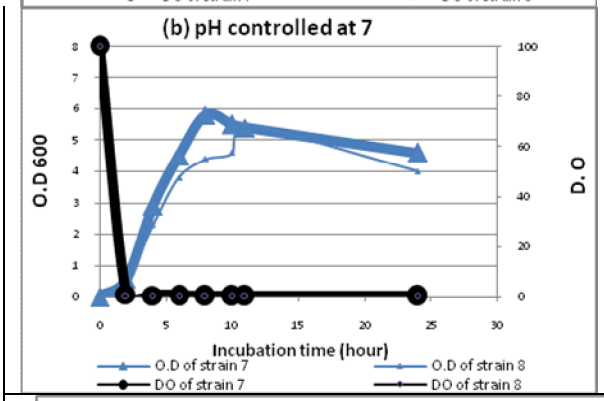
Figure 14. Agarose gel electrophoresis of PCR product of some selected *E. coli* isolates.
Lane 1, Marker; lane 2, positive control (*E. coli* strain BW25113); lane 3, negative control (*E. coli* strain BW25113Δ*yhcP*); lane 4, isolate 9; lane 5, isolate coded 3; lane 6, isolate coded 37; lane 7, isolate code

Table 2. Susceptibilities of the two *E. coli* strains BW 25113 and BW 25113Δ*yhcp* grown at different pHs in a laboratory fermentor (BioFlo 110)* to pHBA as determined by MIC.

pH	MIC (mM) of pHBA against:		Fold difference
	BW25113	BW25113Δ*yhcp*	
6	20	5	4
7	160	20	8
8	320	80	4

*) the produced cells were withdrawn after 6 hours for inoculation of microtitre plates for determination of MIC under similar conditions to that used for cell growth in the fermentor whenever possible





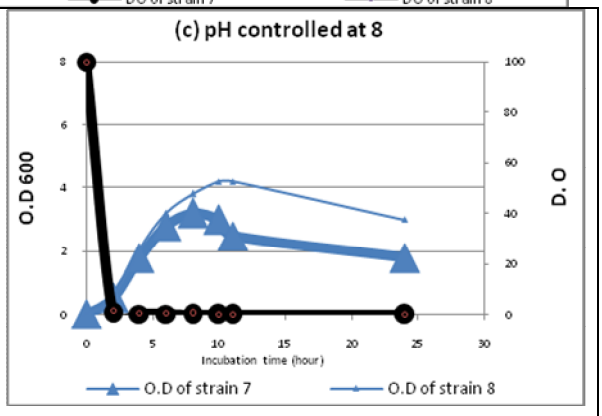
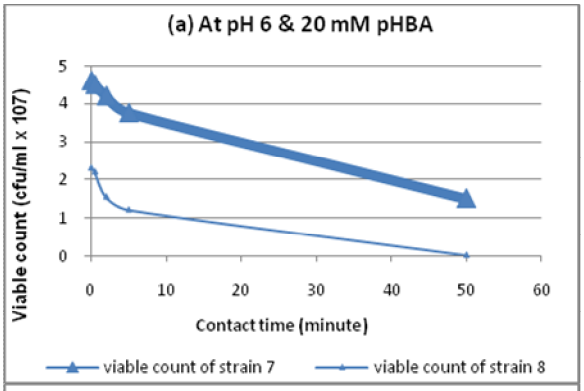
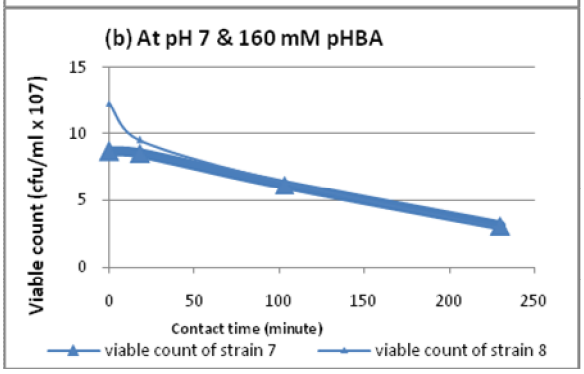


Figure 15. Growth and dissolved O_2 profiles of *E. coli* strains BW25113 and BW25113Δ*yhcP* grown in a laboratory fermentor under controlled pH of 6, 7 and 8. The basal medium containing 2% peptone and 2.6% glucose was used as a growth medium





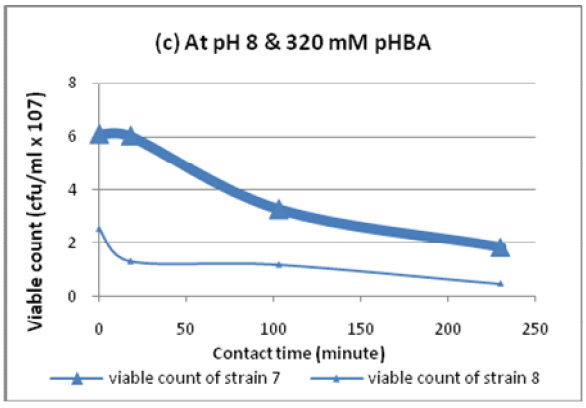


Figure 16**.** Killing Kinetics of *E. coli* strains BW25113 and BW25113Δ*yhcP* by pHBA at different pHs using cells grown in a laboratory fermentor. The cells were grown in a basal medium containing 2% peptone and 2.6% glucose at different pHs. Killing Kinetics was conducted at the same pH used for the cell growth.

production by Sariaslani and Van Dyk, 2007; Amaratunga *et al*., 2000 and Van Dyk *et al*., 2004.

Several variables must be controlled to obtain accurate reproducible results as with any assay system, among these variables that was mentioned by Amsterdam,1996 is pH. The results in Figures 5 & 6 showed that for *E. coli* strains BW25113 and BW25113Δ*yhcp* there was no difference in both growth and pH profiles at the different tested initial pHs in absence of pHBA. The resistant isolate 9 showed similar pH profile but different growth profile to its corresponding sensitive isolate 3.

The susceptibility of the selected isolates/strains to 2.5 mM pHBA at three different initial pH values (5.3, 5.7 and 6.8) was studied. At the initial pH 5.3 both sensitive and resistant isolates/strains showed very high susceptibility. At the initial pH 5.7 there was a clear difference in both the growth and pH profiles between the resistant isolates and their corresponding sensitive isolates as shown in Figures 7 & 8. The susceptibility of the sensitive isolates to pHBA was high at all data points in contrast to the resistant isolates that showed higher growth profiles. At the initial pH 6.8, susceptibility of the resistant isolates was still lower than the corresponding sensitive isolates in most cases however, a considerable increase in the growth profiles was obtained for the sensitive isolates in comparison to their own profiles at the lower initial pH 5.7. The pH profiles for resistant isolates showed differences compared to those of the sensitive isolates at some data points. Increasing the initial pH caused an increase in the resistance of both sensitive and resistant isolates/strains to pHBA. Results showed that pH 6.8 was the best to increase resistance of the isolates/strains to pHBA.

Van Dyk et al., 2004 recorded that the pHBA sensitivity of *E. coli* strain MG1655 (MIC, 100 mM) as compared to the *tolC* mutant (MIC, 56 mM) showed hypersensitivity conferred by the *tolC* mutation. They suggest the presence of one or more pHBA efflux pumps in *E. coli* that utilize the TolC channel. The opening and closing of TolC was shown to be a pH-dependent process, with wild-type TolC closing at low pH (Andersen *et al*., 2002)then the efflux of pHBA is diminished at the low pHs leading to increased effect on the *E. coli* isolates/strains.

At the end of the study of the effect of different environmental fermentation conditions, it can be concluded that, the optimum fermentation conditions for increasing the growth and/or resistance of the *E. coli* isolates to pHBA in shake flasks included: incubation at 37◦C, 320 rpm, agitation and growth at Initial pH 7 or 8.

Several variables must be controlled to obtain accurate reproducible results as with any assay system; among these variables that were mentioned by Amsterdam, 1996 was medium composition and cation concentration.

Treatment of *E. coli* with pHBA resulted in upregulation of *yhcP*, encoding a protein of the putative efflux protein family that contains highly conserved regions or motifs that are indicative of proteins in the Major Facilitator Superfamily (Sariaslani and Van Dyk, 2007) which functions as secondary transporters, catalysing drug–ion (H+ or Na+) antiport (Poole, 2004). That is why Na and K were tested. Results in Figure 9 showed that for isolates number 3, 8, 24, 25 and 34 their susceptibility to pHBA decreased (MIC became 5 mM instead of 2.5 mM) with the addition of sodium chloride to nutrient broth at all concentrations tested (0.5, 1 and 2%). Sodium chloride concentrations change the osmolarity of medium and have a marked effect on the activity of the aminoglycosides and also the ß-lactams shown as decrease in activity and increase in MICs. This effect of osmolarity varies from strain to strain of bacterium (Amsterdam, 1996) this could help understanding the increase in MIC of isolates 3, 8, 24, 25, 34. The same happened with the addition of potassium chloride to nutrient broth at concentrations of 0.5 and 2 while at 1% concentration, isolate no.34 showed even higher resistance. However, the susceptibility of the isolates 7, 9, 15, 20 and 37, which are of a high MIC for pHBA, their susceptibility to pHBA was not affected by addition of any concentration (0.5, 1, 2%) of KCl or NaCl to nutrient broth as shown in Figure 9.

Potentiation by low concentrations of several nonionic macromolecules does not occur (Coates and Richardson, 1973). Moreover, according to Evans, 1964, polysorbate 20 inactivates phenols, phenol derivatives, benzoic acid, pHBA and their esters. Results in Figure 10 showed that for isolates 3, 8, 24, 25 and 34, their resistance to pHBA increased with the addition of tween 80 to nutrient broth at both concentrations tested (1 and 2%) with isolate 34 showing even higher increase in the resistance with 2% tween 80, the MIC values of pHBA increased two fold by the presence of tween 80 and for the isolate 34, such MIC increased 5 fold. However, for isolates 7, 9, 15, 20 and 37 which are of high MIC values for pHBA, the results displayed in the same Figure showed that the susceptibility of these isolates to pHBA was not affected by addition of tween 80 to nutrient broth at concentration of 1%. While the addition of 2% tween 80 to nutrient broth increased the resistance of isolates 7, 9 and 37, their MIC values increased two fold by the presence of tween 80.

Results shown in Figure 11 demonstrate that for isolates 8, 3, 24, 25 and 34: the addition of glucose (1.5%) decreased susceptibility to pHBA of only isolates 3 and 34 while such addition didn't affect the susceptibility of the other isolates. The addition of sucrose (1.5%) to nutrient broth increased the resistance of all these five isolates. The addition of starch (1%) increased the resistance of isolates 3 and 34 while the resistance of isolate 25 sharply decreased (8 fold reduction in MIC). However, for isolates 7, 9, 15, 20 and 37, the results displayed in the same Figure showed that the susceptibility of these isolates to pHBA was not affected by addition glucose (1.5%) or sucrose (1.5%) or starch (1%) to nutrient broth (the MIC remained 5 mM).

Results shown in Figure 12 demonstrate that resistance of all the isolates showed an increase which could be attributed to the fact that in many instances growth will be faster with a supply of organic nitrogen (Stanbury *et al*., 1995). Comparing the MIC values of the pHBA resistant isolates with those of their corresponding sensitive isolates, the following was observed: the overall pattern revealed that a difference in susceptibility to pHBA could be attained with one condition or more for strain 7 versus strain 8 which reached 4 fold, isolate 9 versus 3, isolate 15 versus 24 and isolate 37 versus 34. While such difference in susceptibility became nearly absent with isolate 20 versus 25 (Figure 12).

Results shown in Figure 13 demonstrate that the addition of beef extract to the basal medium in all concentrations tested resulted in an increase in resistance of all the ten tested isolates. They became all of equal MIC of 40 mM except for strain no.8 (BW25113Δ*yhcP*) which showed MIC value of 20 mM with 2 fold difference from strain no.7 (BW25113) with the combination of 2% beef extract and 2.6% glucose.

Results shown in Figure 14 revealed that the *yhcP* gene was detected in both sensitive and resistant wild isolates of natural sources. This may indicate that the resistance wasn't solely dependent on gene acquisition but the regulation of expression is crucial for the development of resistance. Accordingly, the different environmental, physiological and genetic factors affecting the regulation of *yhcP* gene should be studied and controlled for the purpose of increasing bacterial production of pHBA.

In batch culture the pH of an actively growing culture will not remain constant for very long time (Stanbury *et al*., 1995).The results in Figure 15 show that the two strains had similar dissolved oxygen profiles at the different tested pHs (6, 7 and 8) but different growth profiles. Both strains showed higher growth rates at pH 6 and 7 than that at pH 8. The results in Figure 16 showed that the killing kinetics rate of strain BW25113 was less than that of strain BW25113Δ*yhcP* especially in the early periods of exposure to pHBA. This could be due to the fact that *E. coli yhcP* encodes an efflux pump for which pHBA is a substrate. Hence, the absence of this efflux pump results in increased intracellular concentrations of pHBA, which in turn is manifested as the hypersensitive phenotype (Van Dyk et al., 2004) and of course a higher killing kinetics rate. The killing kinetics rates of the two strains were higher at pH 6 than those at pH 7 and 8 showing a remarkable decrease in the number of survivor cells by 50 minutes while at pH 7 and 8 it took a much longer time about 4 hours to give such a decrease. pHBA is a weak acid and the undissociated form, HA, is the active antimicrobial form (Hugo & Russell, 1998) then the low pH (pH 6) caused an increase in the active form of the pHBA and consequently increased effect on both strains noticed by the high kinetics rates but of course the presence of the pump helped strain 7 which showed a relatively lower killing rate in comparison to strain 8. Increasing the pH will cause the weak acid to dissociate thus decreasing the active or diffusible form leading to a slower rate of killing kinetics for both strains, yet a difference between strain 7 and 8 is present due to the presence of the pump in the former strain. The same can be applied for the MIC values of pHBA against the two tested strains at the different tested pH values (Table 2). The MIC against the two tested strains increased by increasing the pH as the acid effect decreased but the obvious difference between the 2 strains (4 and 8 fold) is attributed to the efflux pump. The highest difference in MIC values between the two strains was observed at pH 7 (8 fold differences). This could be attributed to the fact that the uncharged acid form, which is the moiety that diffuses into the cell, is a minor component at neutral pH (Van Dyk et al., 2004).

From this study it can be concluded that the optimum fermentation conditions for increasing the growth and/or resistance of *E. coli* BW25113 to pHBA in a laboratory fermentor include: incubation at 37◦C, 200 rpm agitation, growth in basal medium containing 2% peptone and 2.6% glucose under controlled pH 8.

Further work applying these optimized fermentation conditions on genetically improved *E. coli* strains with active efflux pump may lead to striking results in terms of increased production of pHBA and could provide an effective solution of its toxicity to the producing host bacterial cell which in turn will help to increase production of this molecule for commercial use.

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