Determination of minimum growth inhibitory concentration (MIC) of food acidulants

By

Mr.Thanakorn Sathitwitayakul

ID 4718217

A special project submitted to the Faculty of Biotechnology, Assumption University in part fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology

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ABSTRACT

Acetic, Adipic, Ascorbic, Citric and Sorbic acid, common food acidulant applied in food, have been investigated systematically with regard to their ability to inhibit the growth of reference stain *Escherichia coli* by serial dilution method. 0.57, 0.14, 9.88, 9.93, and 3.46 mg/ml of Acetic, Adipic, Ascorbic, Citric and Sorbic acid correspondingly are minimum concentration that capable to inhibit *E.coli* growth at aqueous solution and without adjustment of pH when concentration of culture is 1.43×10^4 CFU/ml. Minimum Growth Inhibitory Concentration (MIC) of acidulants have been demonstrated using graded concentration technique, 4 series of graded concentration were organize for superiority results. The results of this study suggest a significant antibacterial function of food acidulants with the most significant effective, Adipic acid, among the others. For further study, specific studies of MIC; determination of optimal pH, temperature including affect of food to MIC are recommended.

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Thanakorn Sathitwitayakul

September, 2008

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List of Abbreviations

% (v/v)	For	Percent volume by volume
% (w/v)	For	Percent weight by volume
CFU	For	Colony Forming Unit
Conc.	For	Concentration
Ref	For	Reference
Sol	For	Solution
Rep.	For	Replication
MIC	For	Minimum growth Inhibitory Concentration
g	For	Gram
mg	For	Milligram
ml	For	Milliliter
pH	For	Negative logarithm of photon concentration
pK_a	For	Negative logarithm of dissociation constant
°C	For	Degree Celsius
CRD	For	Complete Randomized Design
cm	For	Centimeter
FDA	For	Food and Drug Administration
FAO	For	Food and Agriculture Organization of United nation
WHO	For	World Health Organization
ppm	For	Part per million

DETERMINATION OF MINIMUM GROWTH INHIBITORY CONCENTRATION (MIC) OF FOOD ACIDULANTS

CHAPTER I

INTRODUCTION

1.1 Background

Microbial infection is the main cause of the food spoilage apart from physical and chemical deterioration of food. Visible microbial growth presents in the form of slimy surface or colonies, and cause degradation of structural components of the food and its package which leads to defection of food product, but the most common manifestation will be chemical products of microbial metabolism (29). They also cause consumer the food borne disease, called food poisoning: it is a general name given to illnesses contracted by consuming contaminated food. Food poisoning outbreaks are often recognized by the sudden onset of illness within a short period of time among many individuals who have eaten one or more foods. In common, Single cases are difficult to identify unless, as in Botulism for example, there are distinct symptoms (17).

There are sought ways to preserve process food; traditionally, food was preserved by use of heat, cold, drying and fermenting. In some case, chemical were used, but it was not until recent years that extensive use was made of such chemical additives (6). Chemical preservatives have been defined by the Food and Drug Administration (1979) as "any

chemical that when added to food tends to prevent or retard deterioration, but does not include common salt, sugars, vinegars, spices, or oils extracted from spices, substances added to food by direct exposure thereof to wood smoke, or chemicals applied for their respective insecticidal or herbicidal properties" (6).

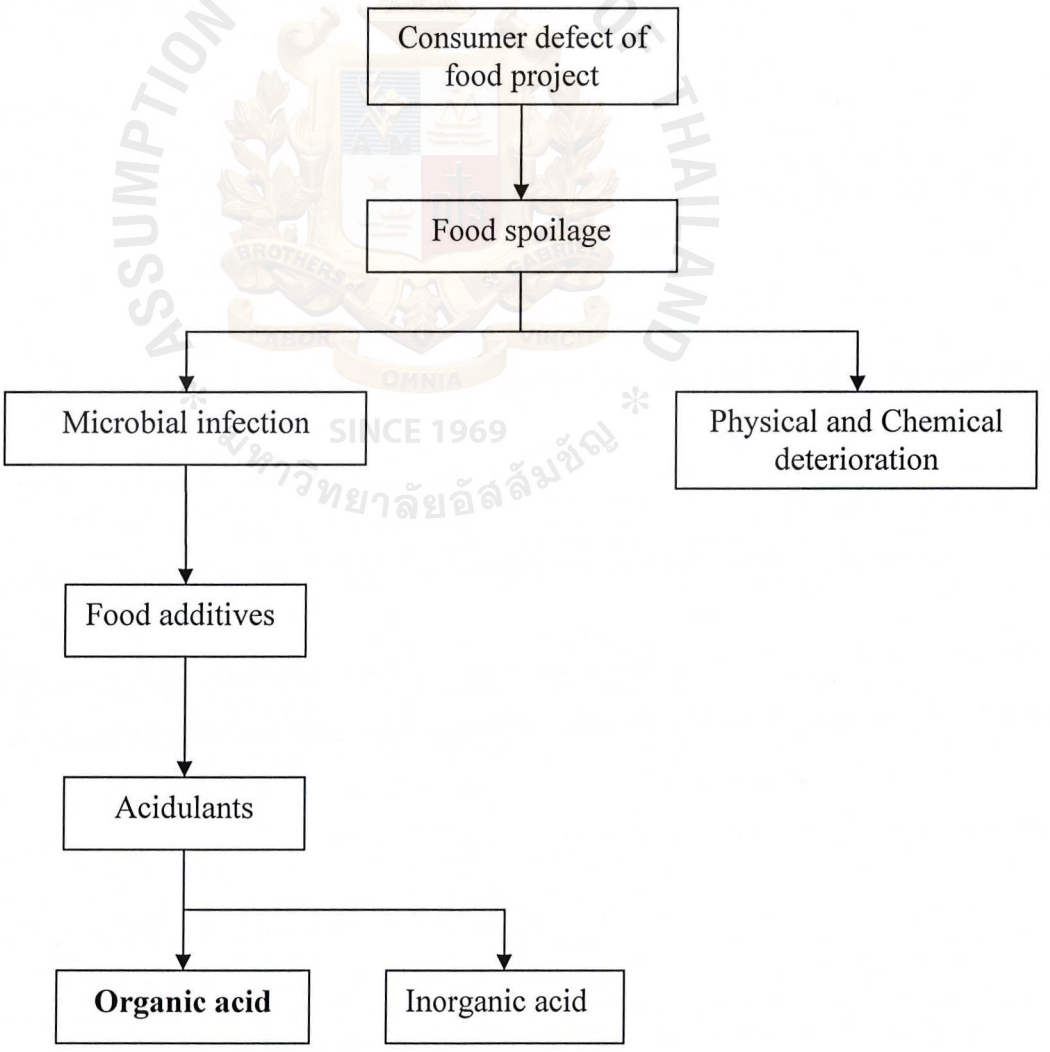
Currently, approximately 30 different compounds can legally be used as antimicrobial in food products (6). Selection of the proper antimicrobial is dependent upon several factors, including the antimicrobial and chemical properties. In the mean of antimicrobial properties of antimicrobial agent, the quantitative assessment of antimicrobial activity is essential of selection of proper antimicrobial agent.

Food acidulants are kind of food preservatives, to accomplish the effective means of preservation; the superior assessment of acidulant concentration, used for food preservation is needed. Thus, the determination of antimicrobial activity of food acidulants is investigated as primary study of their efficacy. In the study five common acidulants are investigated for their quantitative assessment of MIC: Acetic, Adipic, Ascorbic, Citric and Sorbic acid and they efficacy is compared so as to compare the efficacy of them to those of existing antimicrobial efficacy in previous studies (34).

1.2 Objectives

The objectives of this study were to conduct the study of minimum growth inhibitory concentration (MIC) of antimicrobial agent against *Escherichia coli* in the aqueous solution and to compare the efficacy of those antimicrobial agents.

1.3 Conceptual Framework



1.4 Scope

In this study, the MIC of five organic acids which were used as food acidulants or preservatives was determined using serial dilution technique; the study required *E.coli* as culture to investigate MIC of those acids. Organic acids were firstly varied into graded concentration; dilution of the acid in nutrient both, and followed by the incubation of culture with diluted acid at 37°C. Then the growth inhibition of *E.coli* was detected by the mean of visible growth, and MIC of five organic acids was compared using CRD experimental design.

1.5 Hypothesis

1.5.1 Species of antimicrobial agent had different efficacy in ability to inhibit *E.coli* growth.

1.5.2 Citric acid demonstrated highest efficacy among five organic acids

1.6 Benefits of the study

1.6.1 Information about acidulant activity and the comparison of their efficacy.

1.6.2 Primary data for superior assessment of acidulant used in food product.

1.7 Definition of technical terms

1.7.1 Minimum growth Inhibitory concentration (MIC): is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents (2).

1.7.2 Antimicrobial agent : is a substance that kills or inhibits the growth of microbes such as bacteria, fungi, or viruses. Antimicrobial drugs either kill microbes, called microbicidal or prevent the growth of microbes, called microbistatic (3).

1.7.3 Spectophotometer: is a device for measuring light intensity that can measure intensity as a function of the color, or more specifically, the wavelength of light (20).

1.7.4 Optical density: is a unitless measure of the transmittance of an optical element for a given length and wavelength, it can be measure using spectophotometer (1).

1.7.5 Spead plate: is kind of microbial isolation method which is done in aseptic condition. In this technique, the sample is appropriately diluted and a small aliquot transferred to an agar plate. The bacteria are then distributed evenly over the surface by a special streaking technique. After colonies are grown, they are counted and the number of bacteria in the original sample calculated (45).

1.7.6 Steak plate: is the other kind of isolation method; it is done using a bent glass rod. 0.1 ml of bacterial suspension is placed in the center of the plate using a sterile pipet. The glass rod is sterilized by first dipping it into a 70% alcohol solution and then passing it quickly through the Bunsen burner flame. When all the alcohol has burned off and the rod has air cooled, streak the rod back and forth across the plate working up and down several times. Unlike streaking for isolation, you want to backtrack many times in order to distribute the bacteria as evenly as possible. Turn the plate 90 degrees and repeat the side to side, up and down streaking. Turn the plate 45 degrees and streak a third time. Do not sterilize the glass rod between plate turnings. Cover the plate and wait several minutes before turning it upside down for incubation. This will allow the broth to soak into the plate so the bacteria won't drip onto the plate lid (30).

1.7.8 Food acidulants: are excellent antimicrobial agents. They play an important role in the preservation of various food system, either by contrlling the growth of food pathogens by maintaining a appropriate pH or by directly interfering with microbial metabolism (11).

CHAPTER II

LITERATURE REVIEW

Organic acids contribute a variety of functional properties that lead to the enhancement of quality, palatability, nutritive value, and sensory appeal of processed food. Acidulant are excellent antimicrobial agents. They play an important role in the food preservation, either by controlling the growth of food pathogens by maintaining a appropriate pH or by directly interfering with microbial metabolism. In several food, the incorporation of acid into the product at sufficiently high levels can ensure a commercially sterile produce (11). They also stabilize food colors, reduce turbidity, modify melting characteristics, prevent splattering, or enhance gelling (18), (19).

2.1 General function of food acidulants

Food acidulants can be used as pH control agents, as optimum pH is essential for processing or stabilizing a food system. It is particularly essential in gel type products such as gelatin desserts; jams, jellies, and jellied candies (18), (25). Adjustment of pH is essential for optimum development of gel characteristic and gel strength. Even slight variation of pH can have a profound effect on product quality (10).

In some case, they are used as preservative; the acidulants are often added into processed food as their salts form. Acidulants can exert a preservative effect by lowering the pH of a product. An acidic pH often permits a shortening of sterilization time, which in turn results in a better quality, more nutritious product (10).

Certain acidulants, especially the citrates and phosphates, are capable of chelating iron, nickel, manganese, cobalt, chromium, copper, and tin ions. The presence and catalytic action of this ion can often produce undesirable reactions such as discoloration, rancidity, and instability of nutrients (10). Moreover food acidulants play an important role in the enhancement of food flavors. Without them, food such as hard candies, gelatin desserts, carbonated and noncarbonated beverages, jellies, preserves, toppings, and many other products would taste flat. The balance point, the ratio of sweetness to tartness which is called brix acid ratio, can be tuned to state the primary flavor and enhance the secondary flavor notes, which otherwise would be masked by excessive sweetness or tartness (36).

The nutritive value of certain foods is also enhanced by the addition of acidulants when they contain ascorbic acid as one of their components. Unless present in suitable amount, ascorbic acid is preferentially oxidized to dehydroascorbic acid with rapid loss of vitamin activity. High acidity tends to reverse this reaction by maintaining a low redox potential, especially during the initial stages of oxidation (18).

In addition, acidulants can markedly influence the rheological properties of dough when specifically added for this purpose or contained in the leavening agents. They are also used for dispersing food constituents; stabilizing different emulsion systems, as a group they are exceeded in volume of consumption only by the emulsifying agents (18), (35).

2.2 Classification of food acidulants

The different acids used in the food industry can be broadly classified into two groups: inorganic and organic acid (Fig. 2-1). As a group, the organic acids constitute the most widely used acidulants in the food industry (10).

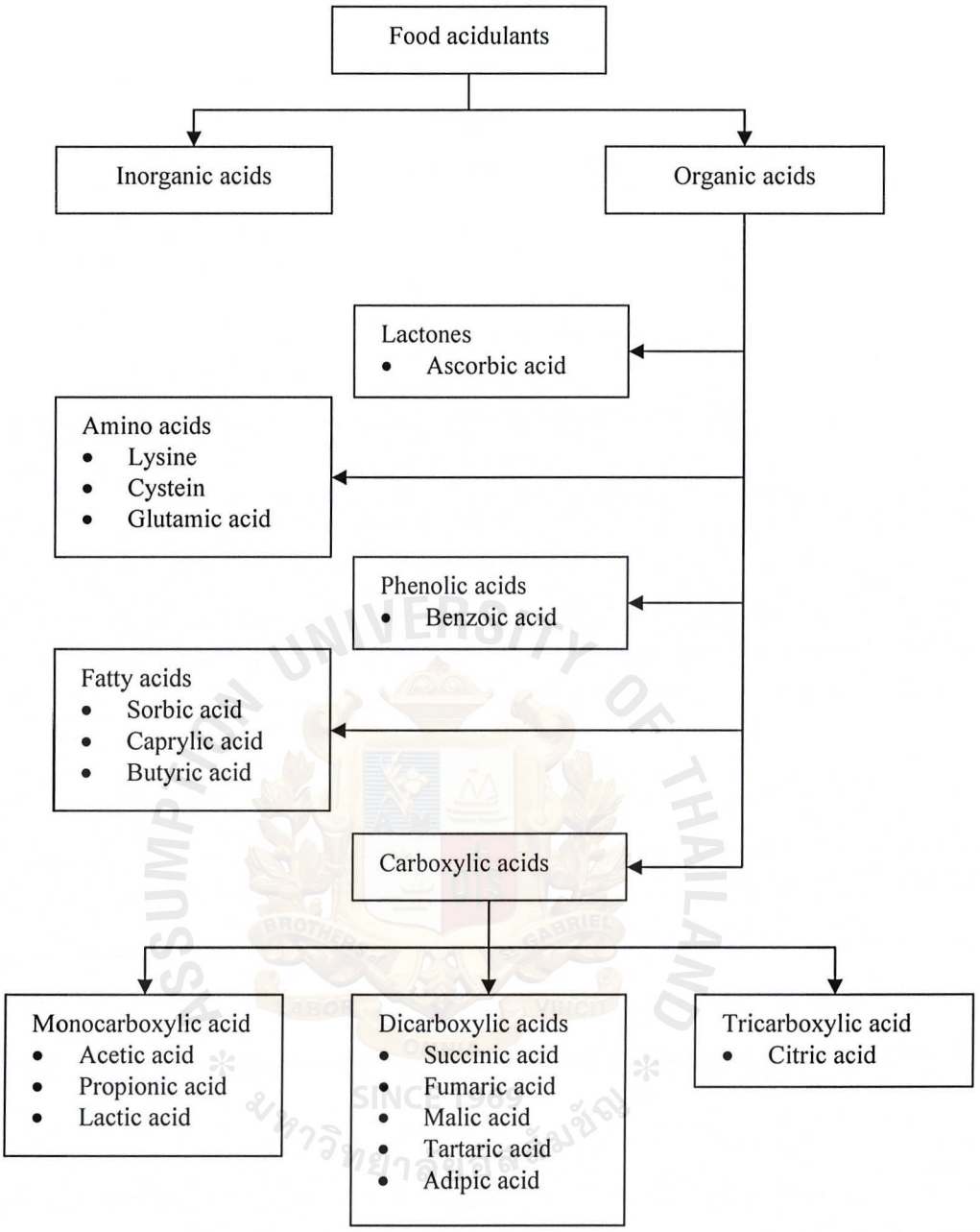


Figure 2-1 Classification of food acidulants (10)

2.3 Organic acid

In general, bacteria are more fastidious and prefer a pH near neutrality (pH 6.5-7.5), but they tolerate a pH range of 4-9. Tolerance of organisms to widely differing pH levels varies naturally, and the pH selects the species or group of microorganisms that will predominate in unaltered food product (12).

Effective use of an acidulant depends upon the dissociation constant pK_a or the pH at which 50% of the total acid is dissociated. The pK_a of organic acids is the main course to discuss the result from the study. Since the no dissociated portion of the molecule is believed to be responsible for the antimicrobial effect (12).

In addition to any antimicrobial effects an acid may possess, the choice of an acidulant may depend upon secondary effects. Acids contribute to the taste and tartness of a product. They may create a synergistic relationship with antioxidants by chelating metal ions. They can control pectin gel formation, aid in the inversion of sucrose, prevent browning, and protect color. Some can be used as chemical leavening agents. The salts of the acids are important in regulating the acidity of the food. (19) According to previous studies, Reference stain of *E.coli* is inhibit by acid accordingly; HCl < citric = malic < acetic < latic, when no adjustment of pH condition of certain acid solution took place (34).

Table 2-1 Dissociation Constants of Organic Acid in Aqueous Solution

Acids	pK_1	pK_2	pK_3
Acetic acid	4.75		
Adipic acid	5.27	5.41	
Ascorbic acid	4.17		
Citric acid	3.14	4.77	6.39
Sorbic acid	4.76		

Ref. (10), (37)

2.3.1 Acetic acid

Acetic acid (CH_3COOH) is a colorless, clear liquid that has a sharp vinegary odor and a burning taste. Its dilute aqueous solution has been used since the earliest recorded human history. The term acetic acid has been introduced by Libavius (1540-1600 A.D.). Kolbe was credited with the preparation of acetic acid from its elements in 1847 (45). In contrast, the literal meaning of vinegar is sour wine.

Virtually all acetic acid produced commercially is made by one of three routes: acetaldehyde oxidation, liquid phase hydrocarbon oxidation, and methanol carbonylation (15), (31), (45). Vinegar is produced from cider, grapes (or wine), sucrose, glucose, or malt by successive alcoholic and acetous fermentations (18).

Vinegar is used as an acidifier, flavor enhancer, flavoring agent, pH control agent, pickling agent, solvent, and for its antimicrobial

properties. It is extensively used in the preparation of salad dressings, mayonnaise, sour and sweet pickles, sauces and Ketchup, cheese, chewing gum, dairy products, baked goods, rendered fats, gravies, and oils. They are also used in the curing of meat and in the canning of certain vegetables (18), (28). Due to its low cost and antimicrobial action, it is often added to infant feeding formulas to replace lactic acid (11).

Moreover, acetic acid is more effective in limiting yeast and bacterial growth than mold growth. It probably works by lowering the pH below the optimum levels for growth. *Acetobacter* and certain lactic acid bacteria can tolerate acetic acid, since they are often associated with the acid in fermented products such as pickles, sauerkraut, and vinegar (11), (13).

Table 2-2 Physical Properties of Acetic Acid

Chemical name	Monocarboxylic acid, ethanoic acid
Molecular formula	CH_3COOH ($C_2H_4O_2$)
Molecular weight	60.06
Appearance	Clear, colorless liquid, pungent odor
Ionization constant, K_i	8×10^{-5}
Melting point	16.7°C
Boiling point	118.1°C
Flash point	43°C (close cup), 57°C (open cup)
Lower explosive level	5.4 vol % at 100°C
Upper explosive level	16 vol % at 100°C
Specific gravity	1.049 at 20°C/4°C
Surface tension	27.57 dyne/cm at 20.1°C
Solubility	Miscible in water, alcohol, glycerol, and ether

Ref: (7), (13), (18), (24)

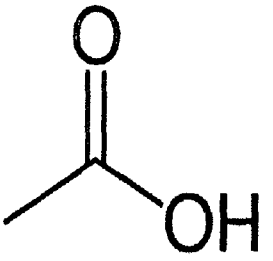


Figure 2-2 Chemical structure of Acetic acid (10)

Since acetic acid occurs naturally in plant and animal tissues and is involved in fatty acid and carbohydrate metabolism as acetyl CoA, and because humans consume about 1g/day acetic acid in vinegar and other foods and beverages, the FAO has set no limit on its acceptable daily intake for human (16).

2.3.2 Adipic acid

Adipic acid is the most important of all the aliphatic dicarboxylic acid commercially, with a worldwide annual production of over 2 million metric tons. Its primary use is in the manufacture off nylon-6,6 the polyamide formed by its reaction with 1,6-hexamethylenediamine. Its uses were subsequently extended to the manufacture of other synthetic fibers such as polyester, acrylic, polyolefin, and other polyamide fiber (10).

The predominant commercial route to adipic acid synthesis is via oxidation of cyclohexane. When a high purity cyclohexanos feedstock is employed, yields of adipic acid range from 1.35 to 1.4 kg/kg cyclohexanos (92-96%). The major byproducts are glutaric acid and succinic acid, produced to the extent of about 6 and 2% of the adipic acid syntesized respectively. Use of an impure cyclohexanos feed, by air oxidation of cyclohexane without cyclohexanos refining, lowers adipic and increases glutaric and succinic acid yield (9).

The Code of Federal Regulations (8) allows the use of adipic acid as a flavoring agent, leavening agent, neutralizing agent, and a pH control agent. The FDA has classified adipic acid as a miscellaneous and general purpose food additive and has set the limitations on its use in different food categories. Adipic acid imparts a smooth, tart taste to foods. In grape flavored products, it is added as supplementary flavor and gives an excellent set to food powders containing gelatin (18), (24).

Table 2-3 Physical Properties of Adipic acid

Chemical name	1,4-Butanedicarboxylic acid, hexanedioic acid
Molecular formula	$COOH(CH_2)COOH(C_6H_{10}O_4)$
Molecular weight	146.14
Appearance	White, monoclinic crystals, odorless with tart taste
Melting point	152°C
Boiling point	337.5°C
Specific gravity	1.36 at 25°C/4°C
Solubility,g/100 ml	1.4 at 20°C, 160 at 100°C in water, very soluble in alcohol

Ref: (7), (9), (24)

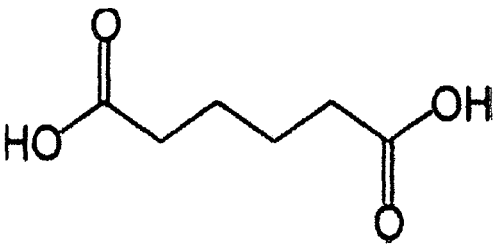


Figure 2-3 Chemical structure of Adipic acid (10)

Adipic acid is a poison by intraperitoneal route, while it is moderately toxic by other routes (24). It is also a severe eye irritant. The FAO/WHO (1967) has established the level causing no toxicological effect of Adipic acid in human at 10,000 ppm in the diet, equivalent to 500 mg/kg body weight per day (10).

2.3.3 Ascorbic acid

Ascorbic acid is widely distributed in the plant and animal kingdom. It is synthesized from simple sugars. D-Glucose is the most important sugar used as a starting material for the synthesis of L-ascorbic acid. It contains the requisite six carbon atoms, some or all of the appropriate chiral centers, and an attractively low cost.

Ascorbic acid occurs as a crystalline powder. It has a pleasant, sharp acidic taste. In impure preparations and in many natural products, it is readily oxidized to dehydroascorbic acid on exposure to air and light,

although it is stable to air when dry. It is readily soluble in water, but insoluble in organic solvents, fats, and oils. It possesses relatively strong reducing power. Its aqueous solutions are rapidly oxidized by air (10).

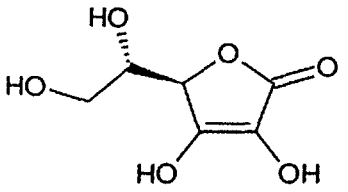


Figure 2-4 Chemical structure of Ascorbic acid (10)

Ascorbic acid is used as an adjunct to meat-curing systems, antimicrobial and antioxidant in foods. It is oxidized in place of other substrates and complements very well as a synergist to other antioxidant. It is also used as an acidulant to adjust pH to prevent the enzymatic browning of fruits and vegetables by polyphenol oxidase enzyme system (13).

Table 2-4 Physical Properties of Ascorbic Acid

Chemical name	L-Xyloascorbic acid, L-3-Ketothreohexuronic acid lactone
Molecular formula	$C_6H_8O_6$
Molecular weight	176.12
Appearance	Monoclinic needle or plate crystals, pleasant, sharp acidic taste
Melting point	190-192°C
Specific gravity	1.65 at 20°C/4°C
Solubility, g/100 ml water	33 at 20°C, 40 at 45°C, 80 at 100°C

Ref: (7), (24)

Ascorbic acid occurs in nature in its reduced form or as the form of L-dehydroascorbic acid, its readily oxidized form. Its biological activity is confined only to its L isomer (18). Plants and mammals except humans, monkeys, and guinea pigs can synthesize ascorbic acid. Those three mammalian species, therefore, require external sources, such as citrus fruits and vegetables, in their daily diet.

2.3.4 Citric acid

Citric acid is a natural constituent and common metabolite of plants and animals. It is the most versatile and widely used organic acid in foods and pharmaceuticals. It is also widely used in several industrial applications to sequester ions, neutralize bases, and act as a buffer (10).

Citric acid was first isolated in the crystalline form from lemon juice (26). It occurs widely in both the plant and animal kingdom. It is also the major acid constituent in the fruits of the citrus family. As the free acid, it is found in the seeds and juices of a wide variety of flowers and seeds (10).

Moreover, citric acid has approved for food uses as an acidifier, curing accelerator, dispersing agent, flavoring agent and as a synergist for antioxidants. It is an important acidulant in dairy product (18). In addition, citric acid is one of the major acidulants in carbonated

beverages, imparting to them a tangy citrus flavor (19), (18). It is also acts as a preservative in syrup and in finished beverage products.

Table 2-5 Physical Properties of Citric acid

Chemical names	2-Hydroxy-1,2,3-propanetricarboxylic acid, β-hydroxytricarballylic acid
Molecular formula	$C_6H_8O_7$
Molecular weight	192.4
Appearance	Colorless, translucent orthorhombic crystals from cold water; anhydrous, colorless translucent monoclinic holohedra crystals, odorless with tart test
Melting point	153°C
Boiling point	Decomposes
Specific gravity	1.665 at 20°C/4°C
Solubility, g/100 ml in water	59.2 at 20°C, 73.5 at 60°C, 84 at 100°C

Ref: (18), (7), (24)

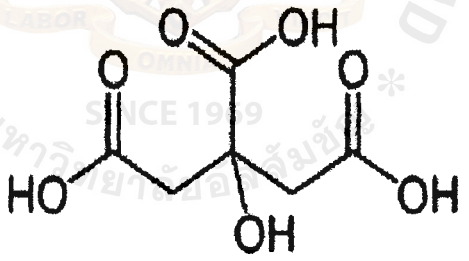


Figure 2-5 Chemical structure of Citric acid (10)

Citric acid is a poison by intrabenous route. It is moderately toxic by subcutaneous and intraperitoneal routes and by ingestion (24). It is a severe eye and moderate skin irritant, and has some allergenic properties.

The FAO (1974) concluded that citric acid and its salts do not constitute a significant toxicological hazard to humans and, hence, no limits were set on their acceptable daily intake in the human diet (10).

2.3.5 Sorbic acid

Sorbic acid is a naturally occurring compound, which is also produced synthetically for application as and antimicrobial agent (39). It was first isolated from the oil of unripened rowan berries in the 1850s, while its antimicrobial properties were discovered in the 1930s and 1940s, with the first patent for use as an antimicrobial agent (38), (40).

The compound, 2,4-hexadienoic, is a straight chain, Trans-Trans, unsaturated fatty acid (CH_3-CH=CH-CH=CH-COOH) with an molecular weight of 112.13 and a highly reactive carboxyl group. Its conjugated double bonds are also reactive. Its solubility in water is 0.15% at room temperature, which increases with increasing of temperature and pH (39)

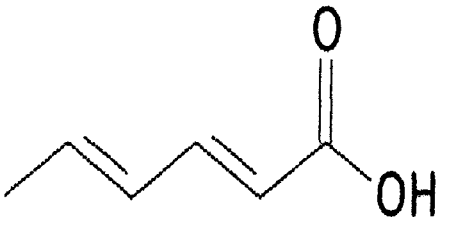


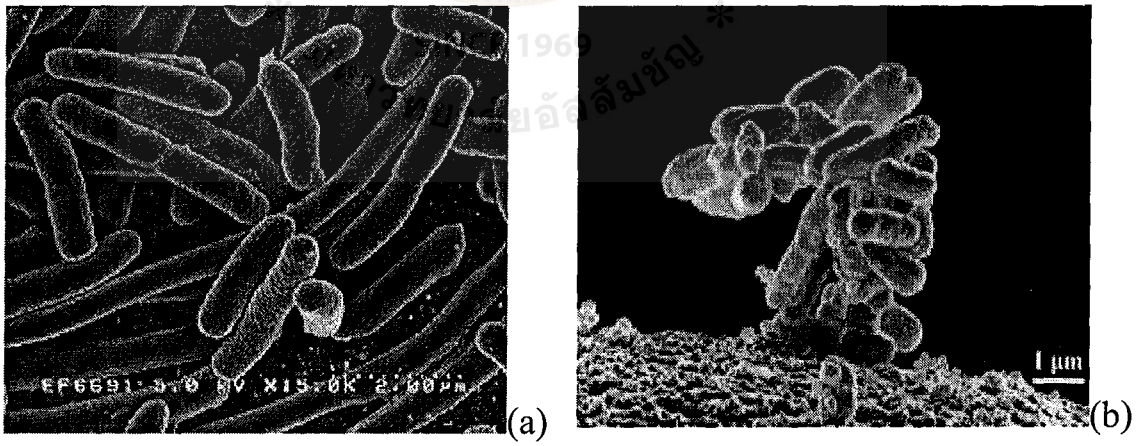
Figure 2-6 Chemical structure of Sorbic acid (10)

Sorbic acid is effective antimicrobial agents against many yeasts and molds as well as bacteria. As yeast inhibitors, the compounds are useful in fermented vegetable products, fruit juices, wines, dried fruits, meat, and fish products (39).

The activity of sorbic acid against microorganisms is a function of synergistic or antagonistic interactions with product composition, pH, water activity, microbial flora, chemical additives, storage temperature, gas atmosphere, and packaging (38). However its activity is 10-600 times less than that of its undissociated acid "sorbate" (41).

WHO has set the acceptable daily intake (ADI) for sorbic acid at 25 mg/kg body weight per day (10).

2.4 *Escherichia coli*



(a) (b)

Figure 2-7 (a) *E. coli* morphology (b) Low-temperature electron micrograph of a cluster of *E. coli* bacteria, magnified 10,000 times. Each individual bacterium is oblong shaped (14)

Escherichia coli is a bacterium that is commonly found in the lower intestine of warm-blooded animals. Most *E. coli* strains are harmless, but some, such as serotype O157:H7, can cause serious food poisoning in humans, and are occasionally responsible for costly product recalls (4), (44). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2 or by preventing the establishment of pathogenic bacteria within the intestine (21), (33)

Table 2-6 Sciencetific classification of *Escherichia coli.*

Domain	Bacteria
Phylum	Proteobacteia
Class	Gamma Proteobateria
Order	Enterobacteriales
Family	Enterobacteriaceac
Genus	Esherichia
Species	*E.coli*

Ref: (14)

E. coli are not always confined to the intestine and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental samples for fecal contamination (14). The bacteria can also be grown easily and its genetics are comparatively simple and easily-manipulated, making it one of the best-studied prokaryotic model organisms, and an important species in biotechnology. *E. coli* was discovered by German pediatrician and bacteriologist Theodor Escherich in 1885 and is now classified as part of the Enterobacteriaceae family of gamma-proteobacteria (14).

2.5 Minimum growth inhibitory concentration (MIC) Determination

In any study of mechanisms of antibacterial action, quantitative assessment of activity under appropriate conditions is an essential part of the initial investigation. Antibacterial action may be either bacteriostatic or bactericidal. Bacteriostatic is the term used to describe the prevention or inhibition of growth by an agent when measured under conditions where growth would normally occur. The effect is reversible such that if the agent is removed or neutralized, the cells will recommence growth and cell division (5). Bactericidal effects occur when bacterial cells exposed to an agent are not recoverable after neutralization of antimicrobial agent. This is due to an irreversible lethal process taking place in the cell (5).

Mechanism of action studies indicate that some antimicrobial agents are primarily bactericidal whilst others may be mainly bacteriostatic. In case of this study, the acidulants exert bacteriostatic effect to culture (22).

In determination of MIC of a bacteriostatic agent, the organism is introduced into the system which contains the acidulant but which also provide optimum nutrients and environmental conditions for growth, followed by an incubation period, usually 18-24 hours (5). The culture is examined either by visually or by other means to assess whether there is

an increase in numbers of viable cells. In laboratory, bacteriostatic assays generally involve either agar diffusion techniques in which inhibition zones are used for assessment of activity or serial dilution method in which the MIC is determined.

For determination of MIC by serial dilution methods which is applied in the study, graded concentrations of the agent are prepared with nutrient broth. For determining MIC, media are inoculated with test organisms, incubated and the MIC determined as the lowest concentration which inhibits visible growth. The accuracy of the end-point will depend on the range of concentrations used. In practice, for initial screening it is usual to employ a series of 10-fold concentration. Once the approximate MIC is known, an arithmetical series of initially not less than eight dilutions is employed (5).

2.6 Relevant research

Lairscey G.R. and Kelly M.T. 1984 (23) studied on an evaluation of a commercial system for the MIC testing of single drugs dehydrated disposable plastic trays. The commercial system was compared with a reference agar dilution method, and 203 clinical bacterial isolates were tested by each method. The results suggest that the Precept system such as serial dilution method may provide a practical and reliable method for MIC determinations of individual antimicrobial agents.

Markus N. *et.al*, 2000 (27) studied the bactericidal activity of Micromolar *N*-Chlorotaurine, which is the main representative of long-lived oxidants found in the supernatant of stimulated granulocytes. They investigated this compound systematically, regarding its antibacterial activity at different physiological concentrations. Moreover bacteria were attenuated after being incubated in N-Chlorotaurine for a sublethal time, as demonstrated with the mouse peritonitis model. They also tested the antimicrobial activity of supernatant of stimulated granulocytes and observed changes in the bacterial cell membrane a cytoplasmic disintegration with transmission electron microscopy. They discussed their result that the acidic condition will enhance NCT activity base upon at low pH the cell membrane will open wider than at higher pH so the agent penetrate into the cell easier and with larger amount therefore the cell metabolism is interfere with higher amount of agent.

Neilson J.W. *et.al,* 1990 (32) studied on a bacteriocin produced by *Pediococcus acidilactici* that had an inhibitory and bactericidal effect on *Listeria monocytogenes* associated with fresh meat. MICs were significantly lower than minimum killing concentrations. When meat was inoculated with *L. monocytogenes*, the bacteriocin reduced the number of attached bacteria in 2 min by 0.5 to 2.2 log cycles depending upon bacteriocin concentration. Meat treated initially with the bacteriocin resulted in attachment of 1.0 to 2.5 log cycles fewer bacteria than that attained with the control. The bacteriocin, after 28 days of refrigerated storage on meat surfaces, was stable and exhibited an inhibitory effect on L. monocytogenes.

Sunisa T., 2006 (43) studied the efficiency of chitosan at variance concentrations in ability to inhibit *Fusarium solani* growth test. The pure chitosan was dissolved in tartaric acid as solutions of chitosan at different concentrations of chitosan. Chitosan agar was form in varieties concentration; 1, 2, 2.5, 3, 3.5, and 4% chitosan solution composed in PDA. Inhibition assay were performed by two methods: spread plate and point plate. Then colony count was performed and diameter of inhibition zone was measured on both methods. The results revealed that the extracted chitosan dissolved in tartaric acid at pH 3.8-5.6. The inhibition of both methods demonstrated that 1-4% chitosam composed could inhibit *Fusarium solani* growth. At 3.5% and 4% of chitosan composed demonstrated the best efficacy in inhibition of *Fusarium solani* growth. Comparing between 3.5% and 4%, 3.5% of chitosan compose has better efficacy. The suggestion was given as 3.5% of chitosan is capable to be used in the improvement of the real field.

Wexler H.M *et.al*, 1990 (46) studied on the designed to estimate the variance components in the determination of the MIC of cefoxitin isolates of the Bacteroides fragilis group. Twenty different organisms were tested, and replicate, trial, and reader variabilities were examined. When the total-variance component was used, if the true MIC was 16 g/ml, then the chance that the observed MIC was between 8 and 32 ug/ml, inclusive, was 95%. For analyses, the isolate ($P = 0.0001$) and reader ($P < 0.03$) effects were significant. The probability of specific MIC preservations for various true MIC (over the range of 16 to 32 μg/ml at 4-,ug/ml increments) was calculated or true MIC of 20, 24, and 28 μg/ml, the probabilities of observing an MIC of 16 or 32 μg/ml (inclusive) were 75, and 62%, respectively. An upward bias was shown to exist in addition

to sources of sizeable variation. The recommendation from recognition of this inherent variability is that ranges of percent susceptibility at various concentrations should be included in reports of in vitro susceptibility studies.

CHAPTER III

RESEARCH METHODOLOGY

This study focused on the study of minimum growth inhibitory concentration (MIC) of antimicrobial agent against *Escherichia coli* and to compare the efficacy of those antimicrobial agents. The function of organic acid as antimicrobial agent which five common acidulants were investigated for their quantitative assessment of MIC: Acetic, Adipic, Ascorbic, Citric and Sorbic acid were compared for their efficacy, and then organic acids were tested for their ability to inhibit *E. coli* growth. In order to achieve this test, the graded concentration of organic acid was separated into four series to achieve three significant number of MIC, the first series refers to initial study of screening, and it was employed with a series of 10-fold dilution which consist of 5 graded concentration: 0.0001, 0.001, 0.01, 0.1, and 1 %w/v or %v/v (apply from Bloomfield S.F:5). Once the estimate MIC was known, the second series was applied, and this series was the arithmetical series which consisted of ten graded concentration of organic acid. Following the second series, the third series was applied when second estimation of MIC was known and it was arithmetical series as well as the forth series. In addition to the determination of estimate MIC from each series, spectrophotometry method was needed to carry out the visible growth of *E.coli*; visible growth should not change if inhibition of acid took place.

3.1 Experiment Locations

All tests: graded concentration of acids preparation, inoculation of culture, and visible growth determination were preformed at Microbiology Laboratory, Faculty of Biotechnology, Assumption University.

3.2 Materials

3.2.1 Chemical reagents

- Adipic acid (Fluka chemika): Laboratory grade
- Citric acid (Carlo Erba Reagent): Laboratory grade
- Sorbic acid (Fluka Chemica): Laboratory grade
- Ascorbic acid (APS Ajex Finechem): Commercial grade
- Acetic acid (J.T. Baker Analyzed): Laboratory grade
- Nutrient broth (see appendix A)
- Peptone water (see appendix A)
- Eosin methylene blue agar (EMB agar) (see appendix A)
- *Escherichia coli* broth (EC broth) (see appendix A)
- BGLB (Brillaint Green Lactose broth) (see appendix A)
- LST (Lauryl Sulfate Tryptose broth) (see appendix A)

3.2.2 Equipment

- Autoclave (Hirayama, Model HA 300MII)
- Incubator (Jouan, EB 280)
- Glassware: Beakers, test tubes, Pipette, and stirring rod
- Micropipette
- Loop
- Alcohol Lamp

3.2.3 Microorganism

- *Escherichia coli* (Reference stain, ATCC25922) was supported by A. Suchawadee Wirathikowit, Faculty of biotechnology, Assumption University.

3.3 Experiment Procedure

The graded concentration of organic acid was separated into four series to achieve three significant number of MIC, the first series refers to initial study of screening, and it was employed with a series of 10-fold dilution. Once the estimate MIC was known, the second series was applied, and this series was the arithmetical series which consisted of ten

graded concentration of organic acid. Following the second series, the third series was applied when second estimation of MIC was known and it was arithmetical series as well as the forth series.

Part A. Preparation of Inoculums

In this study, inoculums size of 1.43×10^4 CFU/ml was used to conduct the study of MIC; the culture with concentration of 1.57×10^8 CFU/ml was diluted to 1.57×10^5 CFU/ml using 10-fold dilution techniques. In order to create the culture with inoculums size of 1.43×10^8 CFU/ml, the refrigerated stock culture was isolated into nutrient broth and was incubate over night. Then it was added into nutrient broth in part B with certain volume to create 1.43×10^4 CFU/ml concentrations. The stock culture was formed by culture purification; the culture, gotten from instructor was purified for *E.coli* using MPN test for *E.coli* (see Appendix A). After the stock culture was grown and was isolated into nutrient broth over night, it was spread on EMB agar of viable cell count.

Part B. Preparation of graded concentration

There are totally 525 tests units as there were four series for each kind of acid, and the study needed triplicate. Each organic acid was weight and dissolved in nutrient broth to the certain volume in volumetric flask. It was furthermore diluted in nutrient broth to create other concentration in the series, by adding x volume of reference acid solution which are the highest concentration of acid in the series where x is decided concentration multiplied by final volume as shown in table 3-1 to 3-6 and divided by reference acid concentration. The 2^{nd},3^{rd}, and the forth series was created by using estimation of MIC of up steam series as highest concentration in series and concentration of acid up steam of MIC test unit as lowest concentration.

Part C. Determination of MIC

After 24 hours incubation of culture from part A in test units from part B, MIC could be determined as the first tube in the series with inhibition of culture; no change in optical density was the result of estimation MIC in each series. To conduct this procedure, initial OD_{600} was measured before incubation and compared to that of after incubation at 37°C for 24 hours.

The Overall procedure was fastidious and complicate; it is needed to show them in the form of table. These tables include graded concentration in the series, final volume of each test unit, and inoculums volume. In the table, volume refer to final of test unit after preparation of grade concentration of acid; graded concentration was not prepared directly from dilution of weighted acid, but it was prepared indirectly by dilution of reference acid solution which was the highest concentration in the series, in nutrient both. Moreover inoculums referred to volume of added culture to achieve concentration of 10^4 CFU/ml.

Table 3-1 Graded concentration of Acetic acid in the series

Series	Concentration (%v/v)									
1	0.0001	0.001	0.01	0.1	1					
2	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
3	0.051	0.052	0.053	0.054	0.055	0.056	0.057	0.058	0.059	0.06
4	0.0541	0.0542	0.0543	0.0544	0.0545	0.0546	0.0547	0.0548	0.0549	0.055

Table 3-2 Graded concentration of Adipic acid in the series

Series	Concentration (%w/v)									
1	0.0001	0.001	0.01	0.1	1					
2	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
3	0.011	0.012	0.013	0.014	0.015	0.016	0.017	0.018	0.019	0.02
4	0.0131	0.0132	0.0133	0.0134	0.0135	0.0136	0.0137	0.0138	0.0139	0.014

Table 3-3 Graded concentration of Ascorbic acid in the series

Series	Concentration (%w/v)									
1	0.0001	0.001	0.01	0.1	1					
2	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
3	0.91	0.92	0.93	0.94	0.95	0.96	0.97	0.98	0.99	1
4	0.981	0.982	0.983	0.984	0.985	0.986	0.987	0.988	0.989	0.99

Table 3-4 Graded concentration of Citric acid in the series

Series	Concentration (%w/v)									
1	0.0001	0.001	0.01	0.1	1					
2	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
3	0.91	0.92	0.93	0.94	0.95	0.96	0.97	0.98	0.99	1
4	0.991	0.992	0.993	0.994	0.995	0.996	0.997	0.998	0.999	1

Table 3-5 Graded concentration of Sorbic acid in the series

Series	Concentration (%w/v)									
1	0.0001	0.001	0.01	0.1	1					
2	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
3	0.31	0.32	0.33	0.34	0.35	0.36	0.37	0.38	0.39	0.4
4	0.341	0.342	0.343	0.344	0.345	0.346	0.347	0.348	0.349	0.35

Table 3-6 Preparation of each acid solution in all series for Acetic acid

Acid Conc, %v/v	Ref Sol Conc, %v/v	Volume of Ref Sol, ml	Volume of NB, ml	Total Volume, ml	Inoculums, ml
0.0001	0.001	1	0	10	1
0.001	0.01	1	0	10	1
0.01	0.1	1	0	10	1
0.1	1	1	0	10	1
1	1	10	0	10	1
0.01	0.1	1	9	10	1
0.02	0.1	2	8	10	1
0.03	0.1	3	7	10	1
0.04	0.1	4	6	10	1
0.05	0.1	5	5	10	1
0.06	0.1	6	4	10	1
0.07	0.1	7	3	10	1
0.08	0.1	8	2	10	1
0.09	0.1	9	1	10	1
0.1	0.1	10	0	10	1
0.051	0.06	5.1	0.9	6	0.6
0.052	0.06	5.2	0.8	6	0.6
0.053	0.06	5.3	0.7	6	0.6
0.054	0.06	5.4	0.6	6	0.6
0.055	0.06	5.5	0.5	6	0.6
0.056	0.06	5.6	0.4	6	0.6
0.057	0.06	5.7	0.3	6	0.6
0.058	0.06	5.8	0.2	6	0.6
0.059	0.06	5.9	0.1	6	0.6
0.06	0.06	6	0	6	0.6
0.0541	0.55	0.541	4.959	5.5	0.55
0.0542	0.55	0.542	4.958	5.5	0.55
0.0543	0.55	0.543	4.957	5.5	0.55
0.0544	0.55	0.544	4.956	5.5	0.55
0.0545	0.55	0.545	4.955	5.5	0.55
0.0546	0.55	0.546	4.954	5.5	0.55
0.0547	0.55	0.547	4.953	5.5	0.55
0.0548	0.55	0.548	4.952	5.5	0.55
0.0549	0.55	0.549	4.951	5.5	0.55
0.055	0.55	0.55	4.95	5.5	0.55

Table 3-7 Preparation of each acid solution in all series for Adipic acid

Acid Conc, %w/v	Ref sol conc, %w/v	Volume of Ref sol, ml	Volume of NB, ml	Total volume, ml	Inoculum, ml
0.0001	0.001	1	0	10	1
0.001	0.01	1	0	10	1
0.01	0.1	1	0	10	1
0.1	1	1	0	10	1
1	1	10	0	10	1
0.01	0.1	1	9	10	1
0.02	0.1	2	8	10	1
0.03	0.1	3	7	10	1
0.04	0.1	4	6	10	1
0.05	0.1	5	5	10	1
0.06	0.1	6	4	10	1
0.07	0.1	7	3	10	1
0.08	0.1	8	2	10	1
0.09	0.1	9	1	10	1
0.1	0.1	10	0	10	1
0.011	0.02	1.1	0.9	2	0.2
0.012	0.02	1.2	0.8	2	0.2
0.013	0.02	1.3	0.7	2	0.2
0.014	0.02	1.4	0.6	2	0.2
0.015	0.02	1.5	0.5	2	0.2
0.016	0.02	1.6	0.4	2	0.2
0.017	0.02	1.7	0.3	2	0.2
0.018	0.02	1.8	0.2	2	0.2
0.019	0.02	1.9	0.1	2	0.2
0.02	0.02	2	0	2	0.2
0.0131	0.014	13.1	0.9	14	1.4
0.0132	0.014	13.2	0.8	14	1.4
0.0133	0.014	13.3	0.7	14	1.4
0.0134	0.014	13.4	0.6	14	1.4
0.0135	0.014	13.5	0.5	14	1.4
0.0136	0.014	13.6	0.4	14	1.4
0.0137	0.014	13.7	0.3	14	1.4
0.0138	0.014	13.8	0.2	14	1.4
0.0139	0.014	13.9	0.1	14	1.4
0.014	0.014	14	0	14	1.4

Table 3-8 Preparation of each acid solution in all series for Ascorbic acid

Acid Conc, %w/v	Ref sol conc, %w/v	Volume of Ref sol, ml	Volume of NB, ml	Total volume, ml	Inoculum, ml
0.0001	0.001	1	0	10	1
0.001	0.01	1	0	10	1
0.01	0.1	1	0	10	1
0.1	1	1	0	10	1
1	1	10	0	10	1
0.1	1	1	9	10	1
0.2	1	2	8	10	1
0.3	1	3	7	10	1
0.4	1	4	6	10	1
0.5	1	5	5	10	1
0.6	1	6	4	10	1
0.7	1	7	3	10	1
0.8	1	8	2	10	1
0.9	1	9	1	10	1
1	1	10	0	10	1
0.91	1	9.1	0.9	10	1
0.92	1	9.2	0.8	10	1
0.93	1	9.3	0.7	10	1
0.94	1	9.4	0.6	10	1
0.95	1	9.5	0.5	10	1
0.96	1	9.6	0.4	10	1
0.97	1	9.7	0.3	10	1
0.98	1	9.8	0.2	10	1
0.99	1	9.9	0.1	10	1
1	1	10	0	10	1
0.981	0.99	9.81	0.09	9.9	0.99
0.982	0.99	9.82	0.08	9.9	0.99
0.983	0.99	9.83	0.07	9.9	0.99
0.984	0.99	9.84	0.06	9.9	0.99
0.985	0.99	9.85	0.05	9.9	0.99
0.986	0.99	9.86	0.04	9.9	0.99
0.987	0.99	9.87	0.03	9.9	0.99
0.988	0.99	9.88	0.02	9.9	0.99
0.989	0.99	9.89	0.01	9.9	0.99
0.99	0.99	9.9	0	9.9	0.99

Table 3-9 Preparation of each acid solution in all series for Citric acid

Acid Conc, %w/v	Ref sol conc, %w/v	Volume of Ref sol, ml	Volume of NB, ml	Total volume, ml	Inoculum, ml
0.0001	0.001	1	0	10	1
0.001	0.01	1	0	10	1
0.01	0.1	1	0	10	1
0.1	1	1	0	10	1
1	1	10	0	10	1
0.1	1	1	9	10	1
0.2	1	2	8	10	1
0.3	1	3	7	10	1
0.4	1	4	6	10	1
0.5	1	5	5	10	1
0.6	1	6	4	10	1
0.7	1	7	3	10	1
0.8	1	8	2	10	1
0.9	1	9	1	10	1
1	1	10	0	10	1
0.91	1	9.1	0.9	10	1
0.92	1	9.2	0.8	10	1
0.93	1	9.3	0.7	10	1
0.94	1	9.4	0.6	10	1
0.95	1	9.5	0.5	10	1
0.96	1	9.6	0.4	10	1
0.97	1	9.7	0.3	10	1
0.98	1	9.8	0.2	10	1
0.99	1	9.9	0.1	10	1
1	1	10	0	10	1
0.991	1	9.91	0.09	10	1
0.992	1	9.92	0.08	10	1
0.993	1	9.93	0.07	10	1
0.994	1	9.94	0.06	10	1
0.995	1	9.95	0.05	10	1
0.996	1	9.96	0.04	10	1
0.997	1	9.97	0.03	10	1
0.998	1	9.98	0.02	10	1
0.999	1	9.99	0.01	10	1
1	1	10	0	10	1

Table 3-10 Preparation of each acid solution in all series for Sorbic acid

Acid Conc, %w/v	Ref sol conc, %w/v	Volume of Ref sol, ml	Volume of NB, ml	Total volume, ml	Inoculum, ml
0.0001	0.001	1	0	10	1
0.001	0.01	1	0	10	1
0.01	0.1	1	0	10	1
0.1	1	1	0	10	1
1	1	10	0	10	1
0.1	1	1	9	10	1
0.2	1	2	8	10	1
0.3	1	3	7	10	1
0.4	1	4	6	10	1
0.5	1	5	5	10	1
0.6	1	6	4	10	1
0.7	1	7	3	10	1
0.8	1	8	2	10	1
0.9	1	9	1	10	1
1	1	10	0	10	1
0.31	0.4	3.1	0.9	4	0.4
0.32	0.4	3.2	0.8	4	0.4
0.33	0.4	3.3	0.7	4	0.4
0.34	0.4	3.4	0.6	4	0.4
0.35	0.4	3.5	0.5	4	0.4
0.36	0.4	3.6	0.4	4	0.4
0.37	0.4	3.7	0.3	4	0.4
0.38	0.4	3.8	0.2	4	0.4
0.39	0.4	3.9	0.1	4	0.4
0.4	0.4	4	0	4	0.4
0.341	0.35	3.41	0.09	3.5	0.35
0.342	0.35	3.42	0.08	3.5	0.35
0.343	0.35	3.43	0.07	3.5	0.35
0.344	0.35	3.44	0.06	3.5	0.35
0.345	0.35	3.45	0.05	3.5	0.35
0.346	0.35	3.46	0.04	3.5	0.35
0.347	0.35	3.47	0.03	3.5	0.35
0.348	0.35	3.48	0.02	3.5	0.35
0.349	0.35	3.49	0.01	3.5	0.35
0.35	0.35	3.5	0	3.5	0.35

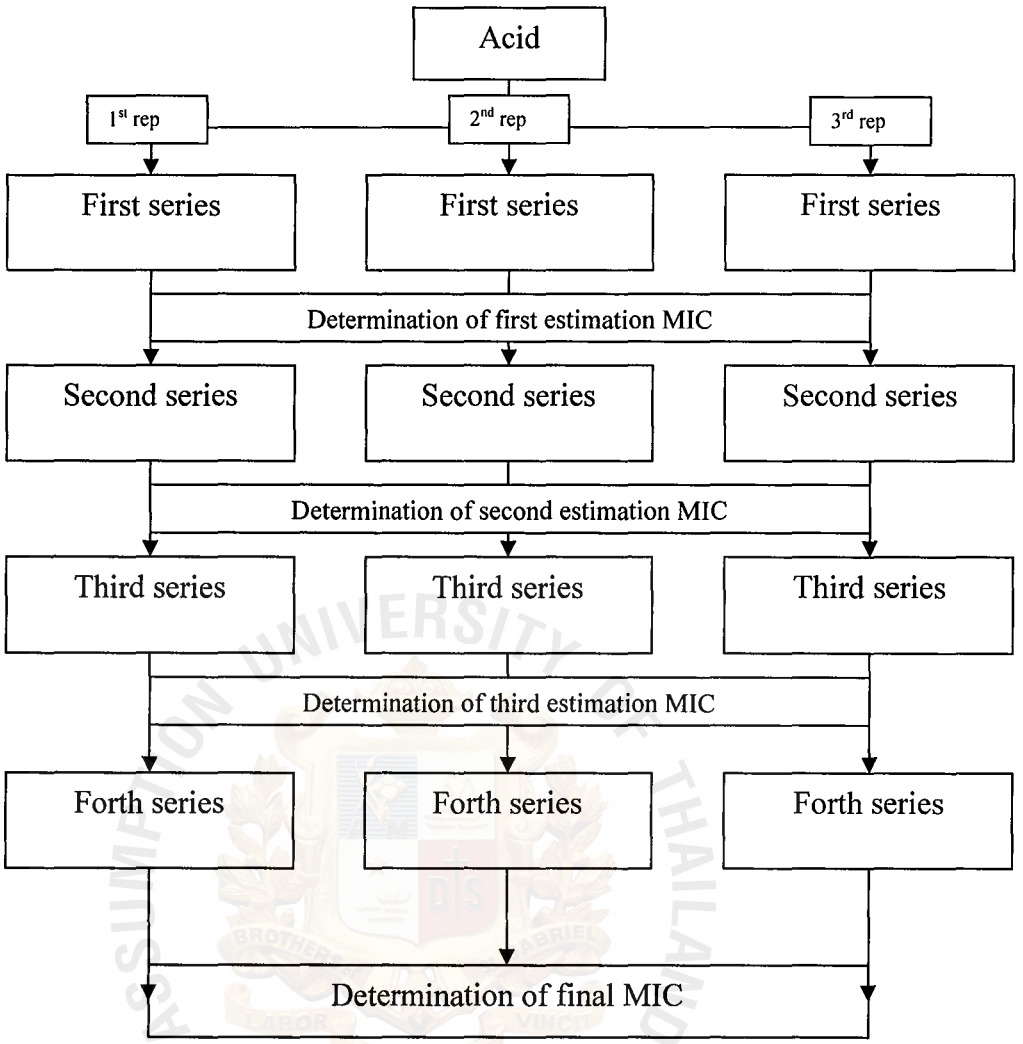


Figure 3-1: The overall study of MIC.

3.4 Data Analysis

For the MIC determination, inhibition of *E.coli* was used as the medium; four series of acid concentration were achieved to investigate the MIC of certain type of acid. MIC of each acid was test for three times (Triplicate) to confirm their result, the final value of certain acid MIC was the average value from these three. Then the difference among acids MIC was compared with the mean of experimental design with F statistic, CRD was applied in this case. In addition, comparison of MIC was conducted using Duncan's multiple rang test. The data were tested for different at 95% confidential level. All the statistical analysis was calculated with software "SPSS 11.0 for window".

3.5 Research duration

Activities / Date	Feb		Mar		Apr		May		Jun		Jul		Aug	
	1	15	1	15	1	15	1	15	1	15	1	15	1	15
Literature review														
Planning/Experimental design														
Laboratory research														
- Planning/Scheduling														
- Preparation of materials														
- Laboratory organization														
Analysis of results														
Presentation														
Report formation														

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Preparation of inoculums

In the purification of *E.coli* culture, the stock culture was purified using MPN method for *E.coli* determination also viable cell was investigated by the mean of spread plate technique with EMB medium.

From the results of MPN, these green colonies on EMB represent *E.coli* (see Appendix D, Figure D1). EMB plate is preferred for viable cell count in this study because of its selective and differential properties. Eosin Y and methylene blue are pH indicator dyes which combine to form a dark purple precipitate at low pH; they also serve to inhibit the growth of most Gram positive organisms so that minimize the change of contamination. Sucrose and lactose serve as fermentable carbohydrate sources which encourage the growth of fecal coliforms and provide a means of differentiating them.

According to the theory, vigorous fermenters of lactose or sucrose will produce quantities of acid sufficient to form the dark purple dye complex. The growth of these organisms will appear dark purple to black. *Escherichia coli*, a vigorous fermenter, often produces a green metallic sheen. Slow or weak fermenter will produce pink

colonies. Colorless colonies indicate that the organism ferments neither lactose nor sucrose and is not a fecal coliform (32).

4.2 Preparation of graded concentration

The graded concentration, gotten from the preparation was shown in table of chapter III. Acid concentration in the series was expressed in the unit of %w/v for solid or powder phase acid; Adipic, Ascorbic, Citric and Sorbic acid. In case of aqueous phase acid; Acetic acid, it was prepared in %v/v.

The graded concentration of acid was prepared indirectly by dilution of reference acid concentration as describe previously. Final volume of these test units were not always 10 ml as shown in previous tables in chapter III. In order to carry out final volume of 10 ml as well as graded concentration in the series, the errors or inaccuracy of the results might occur.

The aim of adding inoculums into each test unit is to dilute the culture to 10-fold: 1 ml of inoculums in 10 ml of aqueous solution. As not all test units achieved final volume of 10 ml so the volume of inoculums being added to test unit is equal to y/10, when y represents the final volume in the test unit. In this step, there are random errors from the rounding of infinite digit number. However this type of error can be

minimized using unbiased rounding or convergent rounding which is identical to the common method of rounding except when the digit following the rounding digit starts with a five and has no non-zero digits after it. The new algorithm is decide which is the last digit to keep, increase it by 1 if the next digit is 6 or more, or a 5 followed by one or more non-zero digits, leave it the same if the next digit is 4 or less, otherwise, if all that follows the last digit is a 5 and possibly trailing zeroes; then change the last digit to the nearest even digit. That is, increase the rounded digit if it is currently odd; leave it if it is already even (5).

4.3 Determination of MIC

Before MIC was determined, the overall study of MIC is needed to be organized and result series was demonstrated in table. In the cast, visible growth of culture was confirmed by mean of optical density.

4.3.1 Determination of Acetic acid MIC

OD_{600} was measured to confirm the visible growth of *E.coli* in certain acid concentration, and the result of optical density was shown in table 4-1 to 4-4, also range of MIC in each series and graded concentration of further series were discuss. In this part, the results were the average value of appendix table C1.

Table 4-1 OD_{600} of acetic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the first series

Concentration of acetic acid % (v/v)	OD_{600}	
	Initial	Final
0.0001	0.097	0.511
0.001	0.035	0.289
0.01	0.027	0.164
0.1	0.020	0.020
1	0.018	0.018

The first determination of MIC was in the range of 0.01-0.1 % (v/v) of acetic acid as the concentration of 0.1 % (v/v) is the minimum concentration in series that demonstrate no change in turbidity. In this case MIC may fall between concentration of 0.01 and 0.1 % (v/v) due to it is not the final determination of MIC. There is a possibility that final MIC would be less than 0.1% but it must be more that 0.01%. Therefore graded concentration for the second series should be ranging of 0.01-0.1 % (v/v), also graded concentration should be prepared in arithmetic order: 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1 % (v/v) in order to cover all concentration in the range according to arithmetic order, and the result was shown in table 4-2. The optical density then was measure to determine the second estimation of MIC.

Table 4-2 OD_{600} of acetic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the second series

Concentration of acetic acid % (v/v)	OD_{600}	
	Initial	Final
0.01	0.061	0.257
0.02	0.043	0.441
0.03	0.024	0.253
0.04	0.028	0.342
0.05	0.028	0.131
0.06	0.051	0.051
0.07	0.040	0.040
0.08	0.009	0.009
0.09	0.019	0.019
0.1	0.043	0.043

The second determination of MIC was in the range of 0.05-0.06 % (v/v) of acetic acid as reason described in previous part. The graded concentrations were prepared to achieve third estimation of MIC and results were shown in the table 4-3;

Table 4-3 OD_{600} of acetic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the third series

Concentration of acetic acid % (v/v)	OD_{600}	
	Initial	Final
0.051	0.031	0.345
0.052	0.033	0.264
0.053	0.036	0.118
0.054	0.038	0.108
0.055	0.039	0.039
0.056	0.042	0.042
0.057	0.045	0.045
0.058	0.045	0.045
0.059	0.047	0.047
0.06	0.046	0.046

The third estimation of MIC was in the range of 0.054-0.055 % (v/v) of acetic acid as reason described in previous part. The graded concentrations were prepared to achieve final MIC determination and results were shown in the table 4-4;

Table 4-4 OD_{600} of acetic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the final series

Concentration of acetic acid % (v/v)	OD_{600}	
	Initial	Final
0.0541	0.046	0.258
0.0542	0.048	0.181
0.0543	0.050	0.176
0.0544	0.051	0.136
0.0545	0.055	0.059
0.0546	0.055	0.055
0.0547	0.054	0.051
0.0548	0.053	0.056
0.0549	0.058	0.058
0.055	0.053	0.053

MIC of acetic was 0.0546 % (v/v) of acetic acid. Then unit was converted form % (v/v) to (g/ml) by mean of acetic acid density, also the final MIC was shown in table 4-22.

4.3.2 Determination of Adipic acid MIC

OD_{600} was measured to confirm the visible growth of *E.coli* in certain acid concentration, and the result of optical density was shown in table 4-5 to 4-8, also range of MIC in each series and graded concentration of further series were discuss. In this part, the results were the average value of appendix table C2.

Table 4-5 OD_{600} of Adipic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the first series

Concentration of Adipic acid % (w/v)	OD_{600}	
	Initial	Final
0.0001	0.033	0.253
0.001	0.094	0.446
0.01	0.037	0.304
0.1	0.039	0.039
1	0.028	0.028

The first determination of MIC was in the range of 0.01-0.1 % (w/v) of Adipic acid as the concentration of 0.1 % (w/v) is the minimum concentration in series that demonstrate no change in turbidity. In this case MIC may fall between concentration of 0.01 and 0.1 % (w/v) as the reason explained in previous part. Therefore graded concentration for the second series should be ranging of 0.01-0.1 % (w/v), also graded concentration should be prepared in arithmetic order: 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1 % (w/v) in order to cover all concentration in the range according to arithmetic order, and the result was shown in table 4-6. The optical density then was measure to determine the second estimation of MIC.

Table 4-6 OD_{600} of Adipic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the second series

Concentration of Adipic acid % (w/v)	OD_{600}	
	Initial	Final
0.01	0.056	0.364
0.02	0.089	0.089
0.03	0.093	0.093
0.04	0.032	0.032
0.05	0.054	0.054
0.06	0.047	0.047
0.07	0.050	0.050
0.08	0.096	0.096
0.09	0.029	0.029
0.1	0.040	0.040

The second estimation of MIC was in the range of 0.01-0.02 % (w/v) of Adipic acid, then graded concentrations were prepared to achieve the third estimation of MIC and results were shown in the table 4-7;

Table 4-7 OD_{600} of Adipic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the third series

Concentration of Adipic acid % (w/v)	OD_{600}	
	Initial	Final
0.011	0.045	0.383
0.012	0.054	0.225
0.013	0.058	0.174
0.014	0.042	0.042
0.015	0.048	0.048
0.016	0.063	0.063
0.017	0.046	0.046
0.018	0.067	0.067
0.019	0.083	0.083
0.02	0.077	0.077

The third estimation of MIC was in the range of 0.013-0.014 % (w/v) of Adipic acid. The graded concentrations were prepared to achieve final determination of MIC determination and results were shown in the table 4-8;

Table 4-8 OD_{600} of Adipic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the final series

Concentration of Adipic acid % (w/v)	OD_{600}	
	Initial	Final
0.0131	0.049	0.224
0.0132	0.063	0.289
0.0133	0.037	0.119
0.0134	0.048	0.053
0.0135	0.043	0.043
0.0136	0.042	0.043
0.0137	0.029	0.029
0.0138	0.043	0.043
0.0139	0.052	0.051
0.014	0.053	0.053

The MIC of Adipic acid is 0.0135 % (w/v) of Adipic acid. The unit was then converted form % (w/v) to (g/ml), also the final MIC was shown in Table 4-22.

4.3.3 Determination of Ascorbic acid MIC

OD_{600} was measured to confirm the visible growth of *E.coli* in certain acid concentration, and the result of optical density was shown in table 4-9 to 4-12, also range of MIC in each series and graded concentration of further series were discuss. In this part, the results were the average value of appendix table C3.

Table 4-9 OD_{600} of Ascorbic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the first series

Concentration of Ascorbic acid % (w/v)	OD_{600}	
	Initial	Final
0.0001	0.019	0.325
0.001	0.034	0.480
0.01	0.040	0.388
0.1	0.056	0.057
1	0.068	0.068

The first determination of MIC was in the range of 0.1-1 % (w/v) of ascorbic acid as the concentration of 1 % (w/v) is the minimum concentration in series that demonstrate no change in turbidity. In this case MIC may fall between concentration of 0.1 and 1 % (w/v) as the reason explained in previous part. Therefore graded concentration for the second series should be ranging of 0.1-1 % (w/v), also graded concentration should be prepared in arithmetic order: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1 % (w/v) in order to cover all concentration in the range according to arithmetic order, and the result was shown in table 4-10. The optical density then was measure to determine the second estimation of MIC.

Table 4-10 OD_{600} of Ascorbic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the second series

Concentration of Ascorbic acid % (w/v)	OD_{600}	
	Initial	Final
0.1	0.040	0.371
0.2	0.044	0.375
0.3	0.051	0.364
0.4	0.055	0.248
0.5	0.039	0.212
0.6	0.054	0.076
0.7	0.048	0.059
0.8	0.046	0.054
0.9	0.054	0.060
1	0.056	0.056

The second estimation of MIC was in the range of 0.9-1 % (w/v) of ascorbic acid, then graded concentrations were prepared to achieve the third estimation of MIC and results were shown in the table 4-11;

Table 4-11 OD_{600} of Ascorbic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the third series

Concentration of Ascorbic acid % (w/v)	OD_{600}	
	Initial	Final
0.91	0.053	0.472
0.92	0.050	0.265
0.93	0.054	0.212
0.94	0.056	0.290
0.95	0.051	0.241
0.96	0.052	0.145
0.97	0.042	0.122
0.98	0.045	0.083
0.99	0.049	0.049
1	0.053	0.053

The third estimation of MIC was in the range of 0.98-0.99 % (w/v) of Ascorbic acid. The graded concentrations were prepared to achieve the final determination of MIC and results were shown in the table 4-12;

Table 4-12 OD_{600} of Ascorbic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the second series

Concentration of Ascorbic acid % (w/v)	OD_{600}	
	Initial	Final
0.981	0.062	0.370
0.982	0.066	0.341
0.983	0.056	0.309
0.984	0.059	0.302
0.985	0.059	0.208
0.986	0.051	0.051
0.987	0.046	0.054
0.988	0.057	0.066
0.989	0.057	0.057
0.99	0.055	0.055

The MIC is 0.989% (w/v) of Ascorbic acid. The unit was converted form % (w/v) to (g/ml), and the result was shown in table 4-22.

4.3.4 Determination of Citric acid MIC

OD_{600} was measured to confirm the visible growth of *E.coli* in certain acid concentration, and the result of optical density was shown in table 4-13 to 4-16, also range of MIC in each series and graded concentration of further series were discuss. In this part, the results were the average value of appendix table C4.

Table 4-13 OD_{600} of Citric acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the first series

Concentration in citric % (w/v)	OD_{600}	
	Initial	Final
0.0001	0.027	0.366
0.001	0.056	0.413
0.01	0.032	0.261
0.1	0.072	0.167
1	0.021	0.021

The first determination of MIC was in the range of 0.1-1 % (w/v) of Citric acid as the concentration of 1 % (w/v) is the minimum concentration in series that demonstrate no change in turbidity. In this case MIC may fall between concentration of 0.1 and 1 % (w/v) as the reason explained in previous part. Therefore graded concentration for the second series should be ranging of 0.1-1 % (w/v), also graded concentration should be prepared in arithmetic order: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1 % (w/v) in order to cover all concentration in the range according to arithmetic order, and the result was shown in table 4-14. The optical density then was measure to determine the second estimation of MIC.

Table 4-14 OD_{600} of Citric acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the second series

Concentration in citric % (w/v)	OD_{600}	
	Initial	Final
0.1	0.048	0.209
0.2	0.036	0.186
0.3	0.050	0.228
0.4	0.039	0.224
0.5	0.035	0.209
0.6	0.047	0.218
0.7	0.040	0.182
0.8	0.031	0.134
0.9	0.028	0.102
1	0.025	0.025

The second estimation of MIC was 0.9-1 % (w/v) of Citric acid. The graded concentrations were prepared to achieve the third estimation of MIC and results were shown in the table 4-15;

Table 4-15 OD_{600} of Citric acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the third series

Concentration in citric % (w/v)	OD_{600}	
	Initial	Final
0.91	0.046	0.336
0.92	0.041	0.247
0.93	0.052	0.210
0.94	0.048	0.288
0.95	0.050	0.185
0.96	0.046	0.183
0.97	0.042	0.089
0.98	0.033	0.080
0.99	0.039	0.057
1	0.044	0.044

The third estimation of MIC was 0.99-1 % (w/v) of Citric acid for. The graded concentrations were prepared to achieve the third estimation of MIC and results were shown in the table 4-16;

Table 4-16 OD_{600} of Citric acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the final series

Concentration in citric % (w/v)	OD_{600}	
	Initial	Final
0.991	0.044	0.190
0.992	0.058	0.147
0.993	0.049	0.049
0.994	0.046	0.129
0.995	0.046	0.047
0.996	0.048	0.048
0.997	0.043	0.043
0.998	0.051	0.051
0.999	0.042	0.042
1	0.050	0.050

MIC of Citric acid is 0.993 % (w/v) of Citric acid. The mean of these three concentrations was investigated and also unit was converted form % (w/v) to (g/ml) and the result was shown in table 4-22.

4.3.5 Determination of Sorbic acid MIC

OD_{600} was measured to confirm the visible growth of *E.coli* in certain acid concentration, and the result of optical density was shown in table 4-17 to 4-20, also range of MIC in each series and graded concentration of further series were discuss. In this part, the results were the average value of appendix table C5.

Table 4-17 OD_{600} of Sorbic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the first series

Concentration in Sorbic % (w/v)	OD_{600}	
	Initial	Final
0.0001	0.025	0.255
0.001	0.037	0.578
0.01	0.022	0.221
0.1	0.050	0.092
1	0.055	0.055

The first determination of MIC was in the range of 0.1-1 % (w/v) of Sorbic acid as the concentration of 1 % (w/v) is the minimum concentration in series that demonstrate no change in turbidity. In this case MIC may fall between concentration of 0.1 and 1 % (w/v) as the reason explained in previous part. Therefore graded concentration for the second series should be ranging of 0.1-1 % (w/v), also graded concentration should be prepared in arithmetic order: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1 % (w/v) in order to cover all concentration in the range according to arithmetic order, and the result was shown in table 4-18. The optical density then was measure to determine the second estimation of MIC.

Table 4-18 OD_{600} of Sorbic acid in nutrient broth with *E.coli* before and after incubation at 37°C for 24 hours of the second series

Concentration in Sorbic % (w/v)	OD_{600}	
	Initial	Final
0.1	0.049	0.581
0.2	0.047	0.527
0.3	0.016	0.431
0.4	0.027	0.027
0.5	0.037	0.037
0.6	0.065	0.065
0.7	0.060	0.060
0.8	0.033	0.033
0.9	0.034	0.034
1	0.067	0.067

The second estimation of MIC was 0.3-0.4 % (w/v) of Sorbic acid. The graded concentrations were prepared to achieve the third estimation of MIC and results were shown in the table 4-19;

Table 4-19 OD_{600} of Sorbic acid in nutrient broth with *E.coli* before and after incubation at 37°C of 24 hours for the third series

Concentration in Sorbic % (w/v)	OD_{600}	
	Initial	Final
0.31	0.054	0.506
0.32	0.055	0.466
0.33	0.043	0.401
0.34	0.046	0.297
0.35	0.047	0.047
0.36	0.040	0.040
0.37	0.053	0.053
0.38	0.045	0.045
0.39	0.051	0.051
0.4	0.064	0.064

The third estimation of MIC was in the range of 0.34-0.35 % (w/v) of Sorbic acid. The graded concentrations were prepared to achieve the final determination of MIC and results were shown in the table 4-20;

Table 4-20 OD_{600} of Sorbic acid in nutrient broth with *E.coli* before and after incubation at 37°C of 24 hours for the second series

Concentration in Sorbic % (w/v)	OD_{600}	
	Initial	Final
0.341	0.052	0.293
0.342	0.058	0.273
0.343	0.047	0.256
0.344	0.050	0.237
0.345	0.043	0.203
0.346	0.038	0.038
0.347	0.040	0.174
0.348	0.034	0.034
0.349	0.045	0.045
0.35	0.048	0.048

MIC of Sorbic acid is 0.346 % (w/v) of Sorbic acid. The unit was converted form % (w/v) to (mg/ml), and the result was shown in table 4-22.

Table 4-21 MIC range from the estimation of each series of organic acids

Acid	MIC range, % (w/v)			Final MIC, %
	1st series	2nd series	3rd series	
Acetic	0.01 - 0.1	0.05 - 0.06	0.054 - 0.055	0.0546
Adipic	0.01 - 0.1	0.01 - 0.02	0.013 - 0.014	0.0135
Ascorbic	0.1 - 1	0.9 - 1	0.98 - 0.99	0.989
Citric	0.1 - 1	0.9 - 1	0.99 - 1	0.992
Sorbic	0.1 - 1	0.3 - 0.4	0.34 - 0.35	0.346

Note: Unit concentration of acetic acid is % (v/v)

The overall result of each acid Minimum growth inhibitory concentration (MIC) was shown in the table;

Table 4-22 MIC of organic acids

Acid	MIC (mg/ml)
Acetic	0.572 b
Adipic	0.135 a
Ascorbic	9.88 d
Citric	9.93 d
Sorbic	3.46 c

Note: Density of acetic acid is 1.049 g/ml

From the CRD type statistic (Table B1), Type of acid significantly affects the inhibition of *E.coli* growth. The results of treatment comparison statistic from Duncan's multiple range tests and LSD (Table B4 and B3) are respect to CRD conclusion and they explain that Adipic, Acetic, Ascorbic, and Sorbic acid have significant different effect in

inhibiting of the of *E.coli* growth, while Ascorbic acid, and Citric acid has similar effect. In such case the efficacy of acid can be arrange: Adipic acid > Acetic acid > Sorbic > Citric acid = Ascorbic acid. According to different pK_a value of each acid, which is the result of negative logarithm of dissociation constant (K_a) and it relates to the pH of organic acid. The acid that has low pK_a; low pH and high K_a will has higher antimicrobial activity that those of higher pK_a. This is the reason why citric acid was predicted to be most efficacies among the others.

As a result of a study, Citric acid is not the best acidulant based upon there are several effects on their ability to inhibit the bacterial growth apart from pK_a; solubility of certain acid. On the other hand Adipic acid express highest ability to adjust pH of aqueous phase among these acidulant.

Some of acid dissociate more than one time such as acid in the group of Dicarboxylic acid or Tricarboxylic acid, but in second and third time it produces much less proton than first time dissociation. Anyway only first time dissociation has significantly effect on efficacy of certain type of acid (34).

On the other hand from the conclusion, given to statistic tests previously, it is believed that there is more than one factor that affects MIC of certain acid such as; pH, solubility of acid in aqueous solution used in the test, etc (27).

Comparison of these acids efficacy to those of existing in the theory; HCl, citric, malic, acetic, and lactic acid - adipic acid has highest efficacy among them, followed by lactic, acetic, malic and citric acid, and HCI in descending order (34). In addition the inhibition of *E.coli* was determined in mean of visible cell growth; the culture is measure at OD_{600} before and after incubation period. The test unit which includes no change in optical density engages the inhibition of *E.*coli.

CHAPTER V

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

From the results of the study, MIC without pH adjustment, as well as the discussions of the results, conclusion is completed according to discussions and the aims of study which are to compare the efficacy of organic acids and to gain the information about MIC of these acids. Since five organic acids were investigated in the study and were compared using CRD type statistic and treatment comparison test was achieve by Duncan's multiple range test as well as LSD, the conclusion can be stated;

1. The efficacy of test organic acid; Acetic, Adipic, Ascorbic, Citric and Sorbic acid are significantly different at 95% confidential level in mean of their ability of growth inhibitor.
2. Adipic acid has highest efficacy among these acids while Ascorbic and Citric efficacy, in the mean of MIC, are relatively the same.
3. The MIC of tested organic acid is ranging from 0 to 10 mg/ml.

5.2 Recommendation of future studies

This study is primary study for antimicrobial activity or efficacy of the acidulant, it regards minimum concentration used in food as a general in such case it doesn't specific to any type of food. In order to carry minimum concentration of acidulant for certain type of food, the main microorganism that causes food spoilage is needed to be investigated. The MIC of acid, capable to inhibit certain microorganism, should be investigated to minimize the amount of acid used in the food, which lead to reduction of capital cost of chemical. On the other hand, some type of food might naturalize the acid compound thus further research is recommended for this field. These recommendations can be demonstrated;

1. Specific study needs risk analysis of contamination as well as maximum possible contaminated inoculums side.
2. pH variation is recommended in further study of food acidulants in order to determine their optimum pH as well as to compare their efficacy in same acidic condition.
3. Acid in fatty acid group is recommended to use in several type of food as they can be flavoring agent as well.
4. Other factors of food that might naturalize organic acid compound should be investigated and controlled.

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APPENDIX A

CUTURE, BROTH, AND CHEMICAL REAGENT PREPARATION

I Culture Preparation - MPN method for E.coli

Material

1. 3 tubes of Lauryl Sulfate Tryptose broth (LST)
2. 3 tubes of EC broth
3. EMB agent
4. Peptone water
5. Kovac's reagent

A. Presumptive test

1. Isolate one loop full of the stock culture into each of three tubes of LST broth
2. Mix by gently rotation
3. Incubate tubes at room temperature (30°C) for 2 days
4. Any tube producing gas is considered as positive for the presence of presumptive *E.coli*

B Confirmation of *E.coli*

1. From each gassing LST tube from the Presumptive test, transfer a loopful of each suspension to a tube of EC broth
2. Incubate EC tube for 24 hours at 45°C and examine for gas production. If negative, incubate future of 48 hours. Use results of this test to calculate MPN of *E.coli.*
3. Steak a loop-full of each gassing EC broth on EMB
4. Incubate for 24 hours at 35°C
5. Examine plates for suspicious *E.coli* colonies, dark centered and flat with metallic green sheen

6. Subculture at least one typical colony from each plate into individuals tubes of peptone water
7. Incubate at 45°C for 18 hours
8. Test culture for indole production by adding 0.2 ml Kovac's indole reagent to each tube. Shake, a positive test is indicated by a red layer on the surface
9. Tubes indicating he presence of indole are recorded as positive for *E.coli*

II Broth preparation procedure

Nutrient broth

Dissolve 3.0 g of beef extract and 5.0 g of peptone in 1 liter of water

Peptone water

Dissolve 0.1 g of peptone in 1 liter of water

Eosin methylene blue agar (EMB agar)

Dilute 5.0 g of lactose, 5.0g of sucrose, 2.0 g of Dipotassium Phosphate, 0.4 g of Eosin Y, 65 mg of Methylene blue, and 13.5 g of Agar to 1 liter of solution. Heat and let it dry in Petri dish.

EC broth

Dilute 20.0 g of casein, 5.0 g of lactose, 1.5 g of Bile salts Mixture, 4.0 g of Dipotassium Hydrogen Phosphate, 1.5 g of Potassium Dihydrogen Phosphate, and 5.0 g of Sodium Chloride to 1 liter of solution.

Brillaint Green Lactose broth

Dilute 10.0 g of peptone, 20.0 g of oxgaII, 10.0 g of lactose, and 13.3 mg of Brilliant Green to 1 liter of solution

Lauryl Sulfate Tryptose broth

Dilute 20.0 g of Tryptose, 5.0 g of lactose, 2.75 g of Dipotassium Phosphate, 2.75 g of Monopotassium Phosphate, 5.0 g of Sodium Chloride, and 0.1 Sodium Lauryl Sulfate to 1 liter of solution.

III Chemical reagent preparation

Certain volume or amount of acid depend on each acid protocol in chapter III was measured and dissolve in NB broth at 37°C.

APPENDIX B

STATISTICAL ANALYSIS

Table B1 Tested variables in CRD type statistic

Between-Subjects Factors

		Value Label	N
Acid	1.00	Acetic acid	3
	2.00	Adipic acid	3
	3.00	Ascorbic acid	3
	4.00	Citric acid	3
	5.00	Sorbic acid	3
REP	1.00		5
	2.00		5
	3.00		5

Table B2 ANOVA table from CRD type statistic with MIC as dependent variable and type of organic acid and replication as independent variable

Tests of Between-Subjects Effects

Dependent Variable: MIC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	28058.802[a]	6	4676.467	92447.70	.000
Intercept	34475.572	1	34475.572	681537.4	.000
REP	.139	2	6.933E-02	1.370	.308
TRT	28058.663	4	7014.666	138670.9	.000
Error	.405	8	5.059E-02		
Total	62534.778	15			
Corrected Total	28059.206	14			

a. R Squared = 1.000 (Adjusted R Squared = 1.000)

Table B3 LSD table (comparison of treatment mean)

Multiple Comparisons

Dependent Variable: MIC

	(I) Acid	(J) Acid	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	Acetic acid	Adipic acid	4.3800*	.18364	.000	3.9565	4.8035
		Ascorbic acid	-93.1067*	.18364	.000	-93.5301	-92.6832
		Citric acid	-93.5067*	.18364	.000	-93.9301	-93.0832
		Sorbic acid	-28.8400*	.18364	.000	-29.2635	-28.4165
	Adipic acid	Acetic acid	-4.3800*	.18364	.000	-4.8035	-3.9565
		Ascorbic acid	-97.4867*	.18364	.000	-97.9101	-97.0632
		Citric acid	-97.8867*	.18364	.000	-98.3101	-97.4632
		Sorbic acid	-33.2200*	.18364	.000	-33.6435	-32.7965
	Ascorbic acid	Acetic acid	93.1067*	.18364	.000	92.6832	93.5301
		Adipic acid	97.4867*	.18364	.000	97.0632	97.9101
		Citric acid	-.4000	.18364	.061	-.8235	.0235
		Sorbic acid	64.2667*	.18364	.000	63.8432	64.6901
	Citric acid	Acetic acid	93.5067*	.18364	.000	93.0832	93.9301
		Adipic acid	97.8867*	.18364	.000	97.4632	98.3101
		Ascorbic acid	.4000	.18364	.061	-.0235	.8235
		Sorbic acid	64.6667*	.18364	.000	64.2432	65.0901
	Sorbic acid	Acetic acid	28.8400*	.18364	.000	28.4165	29.2635
		Adipic acid	33.2200*	.18364	.000	32.7965	33.6435
		Ascorbic acid	-64.2667*	.18364	.000	-64.6901	-63.8432
		Citric acid	-64.6667*	.18364	.000	-65.0901	-64.2432

Based on observed means.

*. The mean difference is significant at the .05 level.

* MIC is in 10^{-1} mg/ml

Table B4 Duncan's multiple range tests

MIC

	Acid	N	Subset 1	2	3	4
Duncan[a,b]	Adipic acid	3	1.3467			
	Acetic acid	3		5.7267		
	Sorbic acid	3			34.5667	
	Ascorbic acid	3				98.8333
	Citric acid	3				99.2333
	Sig.		1.000	1.000	1.000	.061

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 5.059E-02.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = .05.

* MIC is in 10^{-1} mg/ml

APPENDIX C

RAW DATA

Table C1 OD_{600} of Acetic acid in nutrient broth with *E.coli* before and after incubation at 37°C of 24 hours

Concentration of acetic acid % (v/v)	Replication 1		Replication 2		Replication 3		Average	
	Before	After	Before	After	Before	After	Initial	Final
0.0001	0.012	0.487	0.15	0.469	0.13	0.578	0.097	0.511
0.001	0.034	0.271	0.036	0.306	0.035	0.289	0.035	0.289
0.01	0.022	0.159	0.031	0.168	0.027	0.164	0.027	0.164
0.1	0.02	0.02	0.02	0.019	0.02	0.02	0.020	0.020
1	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018
0.01	0.053	0.246	0.068	0.268	0.061	0.257	0.061	0.257
0.02	0.037	0.456	0.049	0.426	0.043	0.441	0.043	0.441
0.03	0.022	0.258	0.026	0.248	0.024	0.253	0.024	0.253
0.04	0.02	0.357	0.035	0.327	0.028	0.342	0.028	0.342
0.05	0.028	0.035	0.027	0.146	0.028	0.213	0.028	0.131
0.06	0.044	0.044	0.057	0.057	0.051	0.051	0.051	0.051
0.07	0.032	0.032	0.048	0.048	0.04	0.04	0.040	0.040
0.08	0.008	0.008	0.01	0.01	0.009	0.008	0.009	0.009
0.09	0.017	0.017	0.02	0.02	0.019	0.019	0.019	0.019
0.1	0.041	0.041	0.045	0.045	0.043	0.043	0.043	0.043
0.051	0.032	0.369	0.03	0.321	0.031	0.345	0.031	0.345
0.052	0.035	0.258	0.031	0.269	0.033	0.264	0.033	0.264
0.053	0.037	0.159	0.035	0.041	0.036	0.154	0.036	0.118
0.054	0.039	0.147	0.036	0.039	0.038	0.137	0.038	0.108
0.055	0.04	0.04	0.037	0.037	0.039	0.039	0.039	0.039
0.056	0.043	0.043	0.041	0.041	0.042	0.042	0.042	0.042
0.057	0.044	0.044	0.046	0.046	0.045	0.045	0.045	0.045
0.058	0.045	0.045	0.045	0.045	0.045	0.045	0.045	0.045
0.059	0.046	0.046	0.047	0.047	0.047	0.047	0.047	0.047
0.06	0.045	0.045	0.046	0.046	0.046	0.046	0.046	0.046
0.0541	0.045	0.159	0.046	0.258	0.046	0.357	0.046	0.258
0.0542	0.048	0.048	0.048	0.231	0.048	0.264	0.048	0.181
0.0543	0.05	0.05	0.049	0.201	0.05	0.278	0.050	0.176
0.0544	0.052	0.052	0.05	0.159	0.051	0.196	0.051	0.136
0.0545	0.056	0.056	0.054	0.061	0.055	0.059	0.055	0.059
0.0546	0.058	0.058	0.052	0.052	0.055	0.055	0.055	0.055
0.0547	0.057	0.059	0.051	0.053	0.054	0.05	0.054	0.054
0.0548	0.054	0.06	0.051	0.051	0.053	0.047	0.053	0.053
0.0549	0.059	0.059	0.056	0.056	0.058	0.058	0.058	0.058
0.055	0.054	0.054	0.051	0.051	0.053	0.053	0.053	0.053

Table C2 OD_{600} of Adipic acid in nutrient broth with *E.coli* before and after incubation at 37°C of 24 hours

Concentration of Adipic acid % (w/v)	Replication 1		Replication 2		Replication 3		Average	
	Before	After	Before	After	Before	After	Initial	Final
0.0001	0.025	0.368	0.015	0.264	0.06	0.128	0.033	0.253
0.001	0.088	0.441	0.099	0.452	0.0935	0.446	0.094	0.446
0.01	0.031	0.298	0.043	0.31	0.037	0.304	0.037	0.304
0.1	0.032	0.032	0.046	0.046	0.039	0.039	0.039	0.039
1	0.023	0.023	0.034	0.034	0.028	0.028	0.028	0.028
0.01	0.051	0.357	0.062	0.428	0.056	0.308	0.056	0.364
0.02	0.083	0.083	0.095	0.095	0.089	0.089	0.089	0.089
0.03	0.086	0.086	0.1	0.1	0.093	0.093	0.093	0.093
0.04	0.027	0.027	0.038	0.038	0.032	0.032	0.032	0.032
0.05	0.048	0.048	0.06	0.06	0.054	0.054	0.054	0.054
0.06	0.042	0.042	0.053	0.053	0.047	0.047	0.047	0.047
0.07	0.044	0.044	0.056	0.056	0.05	0.05	0.050	0.050
0.08	0.089	0.089	0.103	0.103	0.096	0.096	0.096	0.096
0.09	0.024	0.024	0.035	0.035	0.029	0.029	0.029	0.029
0.1	0.034	0.034	0.046	0.046	0.04	0.04	0.040	0.040
0.011	0.048	0.358	0.042	0.586	0.045	0.204	0.045	0.383
0.012	0.05	0.268	0.058	0.135	0.054	0.272	0.054	0.225
0.013	0.054	0.159	0.062	0.249	0.058	0.114	0.058	0.174
0.014	0.045	0.045	0.039	0.039	0.042	0.042	0.042	0.042
0.015	0.049	0.049	0.047	0.047	0.048	0.048	0.048	0.048
0.016	0.059	0.059	0.067	0.067	0.063	0.063	0.063	0.063
0.017	0.049	0.049	0.043	0.043	0.046	0.046	0.046	0.046
0.018	0.068	0.068	0.066	0.066	0.067	0.067	0.067	0.067
0.019	0.079	0.079	0.087	0.087	0.083	0.083	0.083	0.083
0.02	0.08	0.08	0.074	0.074	0.077	0.077	0.077	0.077
0.0131	0.052	0.051	0.043	0.263	0.052	0.357	0.049	0.224
0.0132	0.058	0.264	0.074	0.356	0.058	0.248	0.063	0.289
0.0133	0.039	0.157	0.033	0.033	0.039	0.168	0.037	0.119
0.0134	0.059	0.059	0.026	0.026	0.059	0.074	0.048	0.053
0.0135	0.052	0.052	0.025	0.023	0.052	0.054	0.043	0.043
0.0136	0.056	0.056	0.013	0.013	0.056	0.06	0.042	0.043
0.0137	0.026	0.026	0.035	0.035	0.026	0.026	0.029	0.029
0.0138	0.048	0.048	0.032	0.032	0.048	0.048	0.043	0.043
0.0139	0.059	0.059	0.039	0.039	0.059	0.059	0.052	0.052
0.014	0.056	0.056	0.048	0.048	0.056	0.056	0.053	0.053

Table C3 OD_{600} of Ascorbic acid in nutrient broth with *E.coli* before and after incubation at 37°C of 24 hours

Concentration of Ascorbic acid % (w/v)	Replication 1		Replication 2		Replication 3		Average	
	Before	After	Before	After	Before	After	Initial	Final
0.0001	0.015	0.268	0.019	0.354	0.022	0.352	0.019	0.325
0.001	0.028	0.474	0.039	0.485	0.034	0.48	0.034	0.480
0.01	0.034	0.327	0.046	0.447	0.04	0.39	0.040	0.388
0.1	0.049	0.05	0.063	0.064	0.056	0.057	0.056	0.057
1	0.062	0.062	0.073	0.073	0.068	0.068	0.068	0.068
0.1	0.034	0.365	0.045	0.376	0.04	0.371	0.040	0.371
0.2	0.038	0.369	0.05	0.381	0.044	0.375	0.044	0.375
0.3	0.044	0.357	0.058	0.371	0.051	0.364	0.051	0.364
0.4	0.049	0.242	0.06	0.253	0.055	0.248	0.055	0.248
0.5	0.033	0.247	0.045	0.241	0.039	0.147	0.039	0.212
0.6	0.048	0.05	0.059	0.121	0.054	0.056	0.054	0.076
0.7	0.042	0.045	0.054	0.078	0.048	0.055	0.048	0.059
0.8	0.039	0.042	0.053	0.055	0.046	0.064	0.046	0.054
0.9	0.048	0.052	0.059	0.069	0.054	0.058	0.054	0.060
1	0.05	0.05	0.062	0.062	0.056	0.056	0.056	0.056
0.91	0.032	0.541	0.063	0.381	0.065	0.494	0.053	0.472
0.92	0.024	0.458	0.065	0.279	0.061	0.059	0.050	0.265
0.93	0.033	0.421	0.07	0.164	0.059	0.05	0.054	0.212
0.94	0.023	0.428	0.075	0.366	0.071	0.076	0.056	0.290
0.95	0.036	0.359	0.077	0.327	0.039	0.037	0.051	0.241
0.96	0.041	0.349	0.067	0.046	0.048	0.039	0.052	0.145
0.97	0.032	0.045	0.036	0.259	0.058	0.063	0.042	0.122
0.98	0.039	0.041	0.054	0.055	0.042	0.154	0.045	0.083
0.99	0.048	0.048	0.047	0.047	0.053	0.053	0.049	0.049
1	0.057	0.057	0.047	0.047	0.054	0.054	0.053	0.053
0.981	0.044	0.044	0.062	0.541	0.08	0.524	0.062	0.370
0.982	0.05	0.05	0.066	0.458	0.083	0.516	0.066	0.341
0.983	0.053	0.053	0.044	0.421	0.071	0.454	0.056	0.309
0.984	0.045	0.045	0.066	0.428	0.067	0.433	0.059	0.302
0.985	0.048	0.048	0.069	0.359	0.06	0.217	0.059	0.208
0.986	0.056	0.056	0.047	0.349	0.05	-0.252	0.051	0.051
0.987	0.059	0.059	0.038	0.045	0.041	0.058	0.046	0.054
0.988	0.069	0.069	0.035	0.041	0.067	0.087	0.057	0.066
0.989	0.035	0.039	0.056	0.052	0.079	0.079	0.057	0.057
0.99	0.033	0.033	0.061	0.061	0.07	0.07	0.055	0.055

Table C4 OD_{600} of Citric acid in nutrient broth with *E.coli* before and after incubation at 37°C of 24 hours

Concentration in citric % (w/v)	Replication 1		Replication 2		Replication 3		Average	
	Before	After	Before	After	Before	After	Initial	Final
0.0001	0.019	0.157	0.025	0.587	0.036	0.354	0.027	0.366
0.001	0.05	0.407	0.061	0.418	0.056	0.413	0.056	0.413
0.01	0.026	0.255	0.038	0.267	0.032	0.261	0.032	0.261
0.1	0.065	0.159	0.079	0.141	0.072	0.201	0.072	0.167
1	0.015	0.015	0.026	0.026	0.021	0.021	0.021	0.021
0.1	0.042	0.214	0.053	0.268	0.048	0.146	0.048	0.209
0.2	0.03	0.148	0.042	0.263	0.036	0.148	0.036	0.186
0.3	0.043	0.264	0.057	0.156	0.05	0.264	0.050	0.228
0.4	0.033	0.158	0.044	0.268	0.039	0.245	0.039	0.224
0.5	0.029	0.156	0.041	0.257	0.035	0.213	0.035	0.209
0.6	0.041	0.215	0.052	0.254	0.047	0.186	0.047	0.218
0.7	0.034	0.211	0.046	0.159	0.04	0.175	0.040	0.182
0.8	0.024	0.213	0.038	0.045	0.031	0.145	0.031	0.134
0.9	0.022	0.145	0.033	0.036	0.028	0.125	0.028	0.102
1	0.019	0.019	0.031	0.031	0.025	0.025	0.025	0.025
0.91	0.042	0.145	0.053	0.368	0.044	0.495	0.046	0.336
0.92	0.033	0.129	0.045	0.257	0.046	0.354	0.041	0.247
0.93	0.045	0.134	0.059	0.145	0.052	0.351	0.052	0.210
0.94	0.045	0.286	0.056	0.254	0.042	0.325	0.048	0.288
0.95	0.047	0.215	0.059	0.121	0.044	0.218	0.050	0.185
0.96	0.046	0.195	0.057	0.159	0.035	0.196	0.046	0.183
0.97	0.032	0.081	0.044	0.102	0.051	0.085	0.042	0.089
0.98	0.021	0.042	0.035	0.154	0.043	0.045	0.033	0.080
0.99	0.036	0.039	0.047	0.095	0.033	0.036	0.039	0.057
1	0.035	0.035	0.047	0.047	0.05	0.05	0.044	0.044
0.991	0.035	0.368	0.056	0.056	0.041	0.145	0.044	0.190
0.992	0.048	0.257	0.055	0.055	0.07	0.129	0.058	0.147
0.993	0.049	0.049	0.047	0.049	0.051	0.049	0.049	0.049
0.994	0.041	0.041	0.06	0.054	0.037	0.043	0.046	0.046
0.995	0.044	0.047	0.048	0.051	0.046	0.046	0.046	0.048
0.996	0.032	0.032	0.056	0.056	0.055	0.055	0.048	0.048
0.997	0.019	0.019	0.066	0.066	0.043	0.043	0.043	0.043
0.998	0.056	0.056	0.046	0.046	0.052	0.052	0.051	0.051
0.999	0.026	0.026	0.058	0.058	0.041	0.041	0.042	0.042
1	0.057	0.057	0.066	0.066	0.026	0.026	0.050	0.050

Table C5 OD_{600} of Sorbic acid in nutrient broth with *E.coli* before and after incubation at 37°C of 24 hours

Concentration in sorbic % (w/v)	Replication 1		Replication 2		Replication 3		Average	
	Before	After	Before	After	Before	After	Initial	Final
0.0001	0.015	0.357	0.026	0.258	0.034	0.151	0.025	0.255
0.001	0.031	0.572	0.042	0.583	0.037	0.578	0.037	0.578
0.01	0.016	0.276	0.028	0.288	0.022	0.098	0.022	0.221
0.1	0.043	0.148	0.057	0.069	0.05	0.059	0.050	0.092
1	0.049	0.049	0.06	0.06	0.055	0.055	0.055	0.055
0.1	0.043	0.575	0.054	0.586	0.049	0.581	0.049	0.581
0.2	0.041	0.521	0.053	0.533	0.047	0.527	0.047	0.527
0.3	0.009	0.424	0.023	0.438	0.016	0.431	0.016	0.431
0.4	0.021	0.021	0.032	0.032	0.027	0.027	0.027	0.027
0.5	0.031	0.031	0.043	0.043	0.037	0.037	0.037	0.037
0.6	0.059	0.059	0.07	0.07	0.065	0.065	0.065	0.065
0.7	0.054	0.054	0.066	0.066	0.06	0.06	0.060	0.060
0.8	0.026	0.026	0.04	0.04	0.033	0.033	0.033	0.033
0.9	0.028	0.028	0.039	0.039	0.034	0.034	0.034	0.034
1	0.061	0.061	0.073	0.073	0.067	0.067	0.067	0.067
0.31	0.044	0.369	0.055	0.575	0.062	0.575	0.054	0.506
0.32	0.046	0.357	0.058	0.521	0.061	0.521	0.055	0.466
0.33	0.042	0.354	0.056	0.424	0.032	0.424	0.043	0.401
0.34	0.041	0.358	0.052	0.267	0.044	0.267	0.046	0.297
0.35	0.033	0.033	0.045	0.045	0.062	0.062	0.047	0.047
0.36	0.036	0.036	0.047	0.047	0.036	0.036	0.040	0.040
0.37	0.049	0.049	0.061	0.061	0.048	0.048	0.053	0.053
0.38	0.038	0.038	0.052	0.052	0.045	0.045	0.045	0.045
0.39	0.056	0.056	0.067	0.067	0.031	0.031	0.051	0.051
0.4	0.062	0.062	0.074	0.074	0.056	0.056	0.064	0.064
0.341	0.035	0.454	0.056	0.056	0.065	0.369	0.052	0.293
0.342	0.056	0.411	0.054	0.054	0.063	0.353	0.058	0.273
0.343	0.064	0.398	0.045	0.045	0.033	0.324	0.047	0.256
0.344	0.053	0.391	0.054	0.054	0.044	0.267	0.050	0.237
0.345	0.026	0.299	0.061	0.061	0.042	0.249	0.043	0.203
0.346	0.045	0.354	0.058	0.011	0.011	-0.251	0.038	0.038
0.347	0.054	0.059	0.019	0.012	0.046	0.048	0.040	0.040
0.348	0.035	0.035	0.035	0.035	0.031	0.031	0.034	0.034
0.349	0.051	0.045	0.064	0.07	0.019	0.019	0.045	0.045
0.35	0.062	0.062	0.025	0.025	0.056	0.056	0.048	0.048

Unit Conversion

% (v/v) to % (w/v) of acetic acid

% (w/v) = % (v/v) × Density of acetic acid

Where Density of acetic acid = 1.049 g/ml

% (w/v) to mg/ml

mg/ml = % (w/v) × 10

APPENDIX D

FIGURE IN RESEARCH

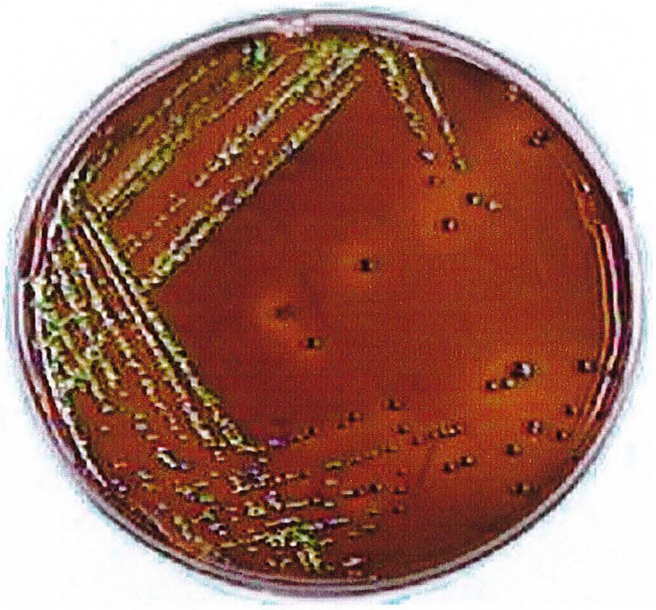


Figure D1 EMB after incubation with *E.coli*

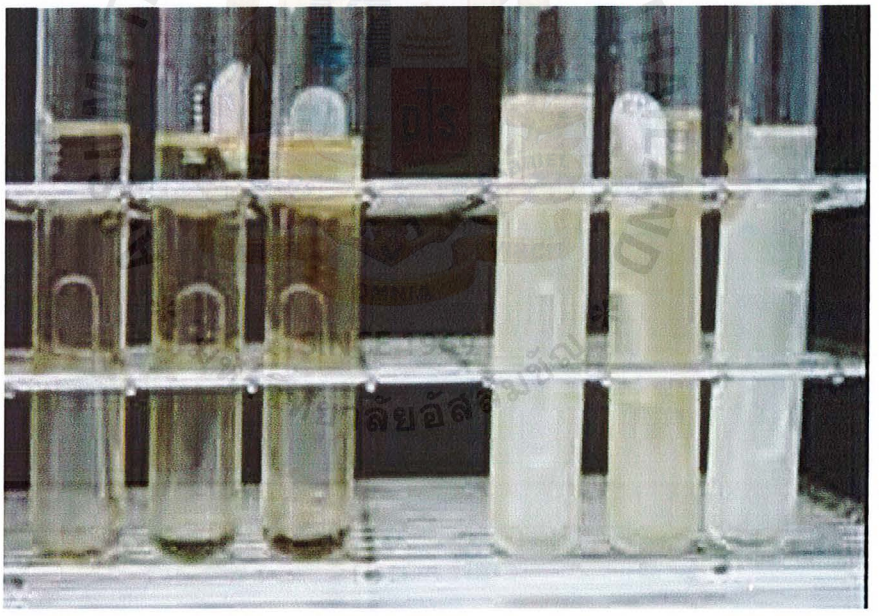


Figure D2 Positive results of presumptive test; (3 tubes on the left) Non inoculated LST (3 tubes on the right) Inoculated LST with *E.coli*

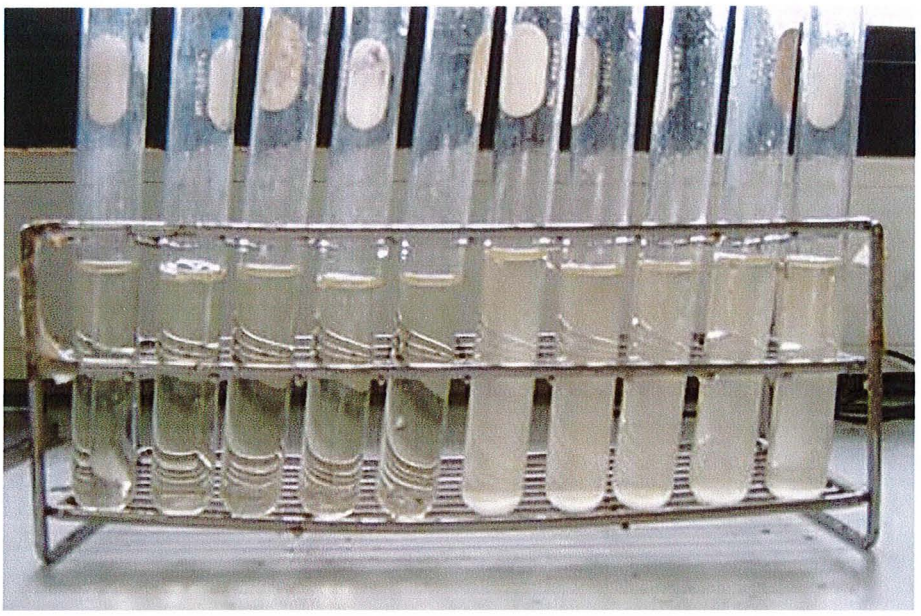
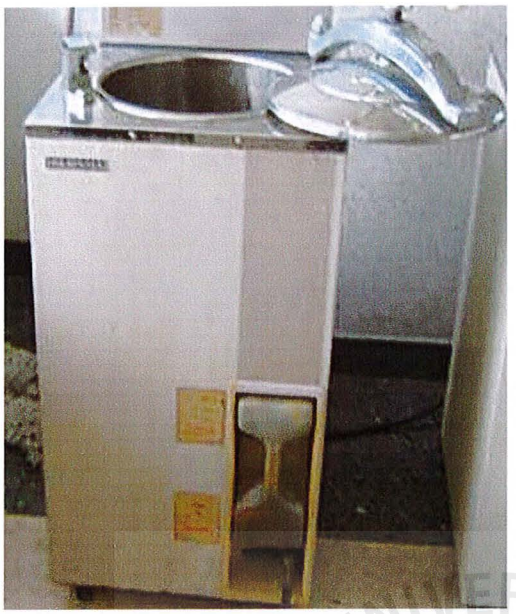


Figure D3 (From left to right) Non inoculated NB completed with acetic, adipic, ascorbic, citric and sorbic acid; from 6th tube; inoculated NB with *E.coli* completed with acetic, adipic, ascorbic, citric and sorbic acid

Figure D4 Spectrophotometer



(a)

(b)

Figure D5 (a) Autoclave (b) incubator