Screening of Lactic acid bacteria suspected to produce 3-Hydroxypropionaldehyde (3-HPA) and bacteriocin from chicken, fish and piglet intestine

By

Miss Avilin Yongperakul

A special senior project submitted to faculty of Biotechnology, Assumption University in part of the fulfillment of the requirements in Bachelor degree of Science Biotechnology , 2010

Screening of Lactic acid bacteria suspected to produce 3-Hydroxypropionaldehyde (3-HPA) and bacteriocin from chicken, fish and piglet intestine

By

Miss Avilin Yongperakul

A special senior project submitted to faculty of Biotechnology, Assumption University in part of the fulfillment of the requirements in Bachelor degree of Science Biotechnology,2010

Special Project

Screening of Lactic acid bacteria suspected to produce 3-Hydroxypropionaldehyde (3-HPA) and bacteriocin from chicken, fish and piglet intestine

By

Miss Avilin Yongperakul

Year 2010

Title: **Screening of Lactic acid bacteria suspected to produce 3-Hydroxypropionaldehyde (3-HPA) from chicken and piglet intestine**

By : Miss Avilin Yongperakul

ID : 491-4264

Advisor : Dr. Tatsaporn Todhanakesem

Level of Study : Bachelor of Science

Faculty : Biotechnology

Major : Agro-Industry Technology

Academic Year : 2010

Advisor Committee: Dr. Viyada Kunathigan

: A. Pijaya Vachajitpan

Tatsaporn Todhanakesem Advisor's Signature

(Dr. Tatsaporn Todhanakesem)

Instructor, School of Biotechnology

ACKNOWLEDGEMENT

I would like to express my gratitude to all those who gave me the possibility to complete this senior project. I want to thank faculty of Biotechnology, Assumption University for giving me opportunity and support to do the senior project and also for all the laboratory instruments provided in order to complete the project.

I am deeply indebted to my advisor Dr. Tatsaporn Todhanakesem, instructor from the Assumption University, for all of her help, suggestions and encouragement throughout the research and writing of this project. I sincerely thank, Miss Nacha Nitistaporn, my senior friend who encouraged me, supported me and gave me a helpful advices to pass through all the obstruct I faced during the research.

Special thank to Dr. Churdchai Cheowteerakul, dean of Biotechnology, for using laboratory equipments for this project. And a special thank Dr. Viyada Kunathigan and A. Pijaya Vachajitpan, committee members in Faculty of Biotechnology, for their time during the project presentation.

Last but not least, I would like to give a special thanks to my parents, family members and my friends for their patient, love, and support which enabled me to complete this senior project.

ABSTRACT

3-Hydroxypropionaldehyde (3-HPA) is an important antimicrobial substance. This substance can be used in many applications as a food preservative or as a therapeutic auxiliary agent. 3-HPA can be produced by conversion of glycerol by glycerol dehydratase found in *Klebsilla spp Clostridium spp., Acetobactor spp.*, and some strains of lactic acid bacteria (LAB) (Hamsupo et al., 2008). In this research we screened merely for the LAB because some LAB can be able to produce 3 HPA and bacteriocin as the product and they are abundant in the animal gastrointestinal tract especially in the piglet and chicken gastrointestinal tract. One hundred and ten colonies were isolated by the plating on the MRS agar with 0.5% $CaCO_3$.Then each colony was tested by using the confirmation test. Among that only forty five colonies passed the confirmation test, further selection indicated that nine colonies were able to inhibit the growth of the *Escherichia coli* on MRS agar supplemented with 80% glycerol and MRS agar. The colonies were then subjected to test for their abilities to produce antimicrobial compound or 3-HPA by paper disc method using two fold dilutions. All isolates displayed the antimicrobial activity against E. coli, of which 4 isolates showed the similar activity to the reference strain, *Lactobacilus reuteri* the Au/ml of the inhibitory activity of both bacteriocin and 3 HPA were in range of 200-300 Au/ml. pH of the cell supernatant both MRS and 80% glycerol were measured. The pH of the cell free MRS solution was ranged in pH of 4-5 where the pH in cell free 80% glycerol solution was ranged in pH of 6-7. Therefore *E.coli* was then grown on the LB media of the pH in range of 4-5 and pH 6-7 to confirm that the inhibitory effects were not created by the acid .The result showed that they could grow on such range of pH which indicated that the clear zone produced around the colonies was not from the inhibition resulted from the acid but from the substances in the solution suspected bacteriocin and 3-HPA.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Recently, there are many industrials shuttered all around the world. All of these industrials have the same aim that is to supply the demand of customer with the product. In order to produce the products that fit the customer's need, many techniques and materials are used. Certainly, industrial chemical is a very important factor for the industrial scale production. So this is where biotechnology comes in and is likely to become a dominant factor in this century (Hinsley, 2005).

For the production of the industrial chemicals, many reactions and chemical precursors are used. 3-Hydroxypropionaldehyde (3-HPA) is considered as a potent antimicrobial substance as well as a precursor for many modern chemicals. Also, it is relatively easy to be converted to a number of larger scale commodity chemicals including acrolein, acrylic acid and 1, 3-propanediol. Moreover it is used for plastic and other polymer production and is considered as a potent antimicrobial substance that is commonly used in the industries (Elsevier B.V., 2004).

3-Hydroxypropionaldehyde can be produced by the conversion of the glycerol by glycerol dehydratase which is found in many kinds of microorganisms such as *Aerobaeter aerogenes, Klebsiella pneumoniae, Enterobacter aerogenes, Clostridium spp.* and *lactic acid bacteria* (LAB)

One good thing about this research is that glycerol is used as the starter, and glycerol can be obtained from many sources because it is organic molecule in human and animals. Moreover, due to the continuously increase of oil price from the oil crisis has stimulated the government and the public to realize the importance of renewable energy. The renewable energy program for Thailand (as planned by DEDE) aims for the biodiesel supply of 600 million liter annually by 2011. The more biodiesel production, the more waste (glycerol) is generated. Conversion of this waste to valuable products not only gives more profit to the industry, but also reduces the treatment or disposal cost. So, the idea to study about the production of 3-

Hydroxypropionaldehyde from various sources by the conversion of gycerol is generated in order to study about the production steps of 3-HPA .*Lactic acid bacteria* is more preferable than the other microorganisms due to the production 3 –HPA and the product is accumulated, whereas in the other microorganisms 3-HPA is an intermediate in the pathway to produce 1, 3 propanediol (Hamsupo et al., 2008).

Among natural biological antagonists, Lactic acid Bacteria have several potential applications. These microorganisms are widely used for the production of fermented foods and are also part of intestinal micro flora. Research reports indicate that LAB represents beneficial health effects in human and animal. They produce some antagonistic compound able to control pathogenic bacteria and undesirable spoilage micro flora because these bacteria have been reported to have strong antimicrobial properties or as bacteriocin. (Hinsley, 2005) .Furthermore, a large number of new bacteriocins produced by lactic acid bacteria have generated interest due to their potential as a safe biopreservatives that could, at least partially, replace chemical preservatives because they comprise a heterogeneous group of proteins showing a narrow or broad antimicrobial activity spectrum against Gram-positive bacteria.

In this study, we will screen Lactic acid bacteria suspected to produce 3-Hydroxypropionaldehyde (3-HPA) and bacteriocin from chicken, fish and piglet intestines from the fresh market in Bangkok in order to isolate the potential strains that are capable of producing 3-HPA and bacteriocin from glycerol and glucose respectively. The research will be an initial work which will support the industrial scale production of antimicrobial compound from waste.

LITERATURE REVIEW

3 HPA

3-Hydroxypropionaldehyde (3-HPA) is a toxic intermediary metabolite in the biological route of 1,3-propanediol biosynthesis from glycerol. 3-HPA accumulated in culture medium would arouse an irreversible cessation of the fermentation process of *Lactobacillus reuteri.* The role of substrate (glycerol) on 3-HPA accumulation in aerobic fermentation was reported (Elsevier B.V., 2004)). The 1, 3-Propanediol oxidoreductase and glycerol dehydratase, two key enzyme catalyzing reactions of 3-HPA formation and consumption are sensitive to high concentration of 3-HPA. When the concentration of 3-HPA increases to a higher level in medium, the activity of 1,3-propanediol oxidoreductase in cell decreases correspondingly, which led to decrease of the 3-HPA conversion rate, then the 3-HPA concentration increasing is accelerated furthermore. 3-HPA accumulation in culture medium is triggered by this positive feedback mechanism(Van Holde and Aderm, 2008)). The level of 3-HPA in culture medium could be controlled by the substrate (glycerol) concentration, and lower level of glycerol could avoid 3-HPA. Glycerol will be converted to 3-HPAby dehydrase then 3-HPA will be further converted to 1, 3-Propanediol by reductase.3-HPA itself, as intermediate chemical substance can be further converted to various chemicals: 3-Hydroxypropionic Acid, Acrolein, Acrylic Acid.

3-hydroxypropionaldehyde (3-HPA) from glycerol by which is the renewable resource the microorganism in an aqueous nutrient medium containing glycerol and a compound that causes 3-HPA to be accumulated by blocking the conversion of 3-HPA to trimethylene glycol. This process is particularly useful for the production, from renewable resources, of acrylic acid, an industrially important polymerizable monomer used in the manufacture of synthetic polymers and plastics and which is presently derived from fossil fuel sources. Moreover, in the field of food industry, 3-HPA-producing *Lactobacillus reuteri* is used as a probiotic .Also antimicrobial 3-HPA has potential as medicine, precursor for several important petro-derived chemicals and antimicrobial compound in food ,therefore some certain types of food

borne pathogen for instance *E.coli* , as one of the main food borne pathogens, can be killed by the 3-HPA due to its antimicrobial activity which is very beneficial in the field of food processing industries (Hurlbert, 1008)

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strain(s). They are typically considered to be narrow spectrum antibiotics, though this has been debated. They are phenomenologically analogous to yeast and paramecium killing factors, and are structurally, functionally, and ecologically diverse. Bacteriocins were first discovered by A. Gratia in 1925. He was involved in the process of searching for ways to kill bacteria, which also resulted in the development of antibiotics and the discovery of bacteriophage, all within a span of a few years. He called his first discovery a *colicine* because it killed *E.coli* (Espinosa and Gonsalez, 2002)

Bacteriocins are of interest in medicine and are attractive focus for drug development because they are made by non-pathogenic bacteria that normally colonize the human body, activate against most pathogens and are not toxic to human cells. Loss of these harmless bacteria following antibiotic use may allow opportunistic pathogenic bacteria to invade the human body. Currently research is under way to improve the efficacy of bacteriocins by genetic manipulation and to enable their production in non-native hosts (Schrechen et al., 1997)

Bacteriocins have also been suggested as a cancer treatment. They have shown distinct promise as a diagnostic agent for some cancers, but their status as a form of therapy remains experimental and outside the main thread of cancer research. Partly this is due to questions about their mechanism of action and the presumption that anti-bacterial agents have no obvious connection to killing mammalian tumor cells. Some of these questions have been addressed. In the long quest for medical applications, bacteriocins have also been tested as AIDS drugs. Some bacteriocins have been employed in food preservation and processing, none have been applied directly as medicine. Recent experiments demonstrate the *in vivo* activity of bacteriocins, the potential importance of bacteriocins as antibiotics, and the role that bacteriocins play

in antibiotic resistance. Meanwhile, several kinds of bacteriocins have been proposed for applications in gastrointestinal microbiology, as well as for the use of probiotics to reduce dental caries and improve oral hygiene.

E. coli ATCC 25922

E. coli ATCC 25922 is a smooth strain commonly used in antibiotic susceptibility testing; it is clinical strains isolated from patients with complicated urinary tract infections (Carprette, 2005).

Table 1: Characteristics of *Escherichia coli* ATCC 25922

Organism:	*Escherichia coli* (Migula) Castellani and Chalmers
Growth condition:	ATCC medium18: Trypticase soy agar **Temperature :** 37.0°C **Atmosphere :** Aerobic
Isolation:	clinical isolate

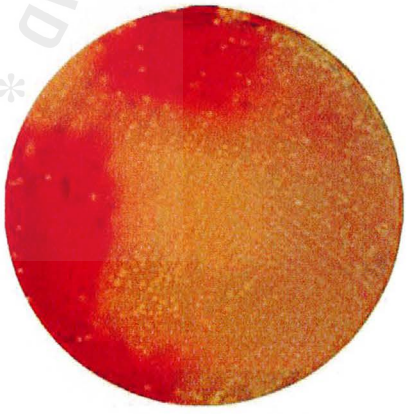


Figure 1: Morphology of *Escherichia coli* ATCC 25922 on XLD AGAR (XYLOSE LYSINE DESOXYCHOLATE AGAR)

From several studies and the researches, the screening of microorganism producing 3-Hydroxypropionaldehyde (3-HPA) and bacteriocins were done. However the screening out of new strains that are capable to produce 3-HPA and bacteriocin still represent a great interest. Therefore, this project lactic acid bacteria were screened to test the ability to produce 3-HPA and the bacteriocins from the chicken,

fish gastrointestinal tract and pig intestine from the fresh market in Bangkok. This project will be benefit in term of medical innovation.

Lactobacillus reuteri

Lactobacillus reuteri is a Gram-positive bacterium that naturally inhabits the gut of mammals and birds. First described in the early 1980s, some strains of *L. reuteri* are used as probiotics. BioGaia AB in Sweden owns several commercially important strains and a large number of different patents for commercial usage of *L. reuteri*.

Lactobacillus reuteri	
Scientific classification	
Kingdom:	Bacteria
Division:	Firmicutes
Class:	Bacilli
Order:	Lactobacillales
Family:	Lactobacillaceae
Genus:	*Lactobacillus*
Species:	***L. reuteri***
Binomial name	
Lactobacillus reuteri	

Scientific Classification of *Lactobacillus reuteri*

L. reuteri producing an anti-microbial agent

The late 1980s, Walter Dobrogosz, Ivan Casas, and their colleagues discovered that *L. reuteri* produced a novel broad-spectrum antibiotic substance via the organism's fermentation of glycerol. They named this substance "Reuterin" Reuterin is a multi-compound dynamic equilibrium (HPA system, HPA) consisting of 3-hydroxypropionaldehyde, its hydrate, and its dimer. At concentrations above 1.4 M, the HPA dimer was predominant. However, at concentrations relevant for biological systems, HPA hydrate was the most abundant, followed by the aldehyde form.

Reuterin has been found to inhibit the growth of some harmful Gram-negative and Gram-positive bacteria, along with yeasts, fungi, and protozoa. Naturally, a gut organism capable of fighting off other, harmful gut organisms was of great interest. Researchers found that *L. reuteri* can indeed secrete sufficient amounts of reuterin to cause the desired anti-microbial effects. Furthermore, since about 4-5 times the

amount of reuterin is needed to kill "good" gut bacteria as "bad", this would allow *L. reuteri* to remove gut invaders while keeping normal gut flora intact.

Some studies have called into question whether or not reuterin production is essential for *L. reuteri* 's health-promoting activity. However, the discovery that it naturally produces an antibiotic substance was nevertheless important, as it has led to a great deal of further research on *L. reuteri*. In fact, in early 2008 it was confirmed that *L. reuteri* is capable of producing reuterin in the gastrointestinal tract, and that this improves its ability to inhibit the growth of *E. coli*.

Antifungal product from an LAB species is reuterin produced by *Lactobacillus* .Reuterin is a by-product of the reduction of glycerol which is an energy yielding pathway (Ree, 2004) As well as toxicity towards a wide range of Gram-negative and Gram-positive bacteria, it has been claimed to be equally effective against lower eucaryotic genera of yeasts and fungi such as *Candida, Torulopsis*, *Saccharomyces*, *Saccharamycoides*, *Aspergillus* and *Fusarium*.

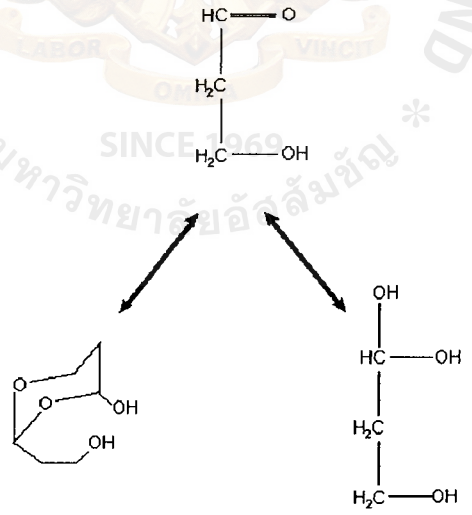


Figure 2. Reuterin, an antimicrobial from *Lactobacillus reuteri*, exists in three forms in aqueous solution (from Talarico & Dobrogosz, 1990)

L. reuteri is unable to grow on glycerol as sole carbon and energy source hence; glycerol is used as an alternative hydrogen acceptor during growth on available carbohydrates. Thus, glycerol is converted to reuterin and 1, 3-propanediol (1, 3-PDL), both products with interesting industrial applications. These compounds are commonly produced by using resting cells in two-step fermentation processes.

Reuterin also have the ability to effect the growth of other microorganisms. Reuterin effects the growth of *Salmonella* spp and effect the lag time of single cells of *Listeria innocua* grown on the surface of Brain Heart Infusion Agar by increasing both the lag time of the cells and its variance. Furthermore, for a large proportion of cells, reuterin completely prevented the cell division within the time of observation. Reuterin in combination with low pH inhibited the cell division even more efficiently.

OBJECTIVE

1. To screen the potential Lactic acid bacteria those are suspected to produce 3-Hydroxypropionaldehyde (3 HPA) and bacteriocin from animal gastrointestinal tract: chicken ,fish and pig intestines.

2. To test the effect of the new screening strains against *Escherichia coli* ATCC 25922

METHODS

1. Isolation of Lactic acid bacteria

Lactic acid bacteria were isolated from chicken, piglet and fish intestines from the fresh market in Bangkok. Isolation was done by the mean of serial dilution from 10^{-1} to 10^{-9} in 0.1% peptone. The enrichment method was introduced for isolation of lactic acid bacteria by culturing serial dilution from 10^{-1} to 10^{-9} in 0.1% peptone overnight before plating, then plated on the MRS + 0.5% $CaCO_3$ and incubated at 37 °C for 2 days (Prasertsub and Methacanon, 2002).

Acid producing bacteria showed the clear zone on MRS + 0.5% $CaCO_3$ which refers as Lactic acid bacteria.

2. Confirmation of lactic acid bacteria

All the positive colonies which represented the clear zone on MRS + 0.5% $CaCO_3$ were transferred onto the MRS +20% glycerol and incubated at 37 °C for 2 days tested on the following confirmation tests.

All positive colonies which represented the clear zone on the MRS +20% glycerol in order to characterize whether the colonies represented in the plate are real lactic acid bacteria by showing the positive result on the confirmative test : Catalase test, gram staining, morphological observation, spore staining, and motility test

Table 2: Lactic acid bacteria characteristics on each test

Test	Characteristic of lactic acid Bacteria
Catalase test	No gas bubble

Gram staining	Gram positive
Morphology observation	Rod shape/cocci
Spore staining	Non spore forming
Motility test	Non motile.

3. *Escherichia coli* preparation

E.coli culture was grown on LB agar and then transferred to tube containing 5 ml of LB broth, incubated overnight. One millimeter of the *E.coli* from the previous tube was transferred to the 250 ml Erlenmeyer flask containing 50 ml of LB agar.the culture were shaken at180 rpm overnight.

Before overlaying on the MRS agar and MRS +20% glycerol agar for the determination of the Antimicrobial spectrumof the candidates over the growth of *E.coli* , 1 ml of *E.coli* from the shake flask was measured the OD value at 600 nm then *E.coli* from the same flask was mixed with LB +0.7% agar when number of the colonies is around 1×10^4 CFU/ml which takes approximately 5 hours for the *E.coli* to grow to that CFU/ml.

4. Determination of Antimicrobial spectrum

All screening candidates were determined on theire antimicrobial activities by streaking the culture on MRS agar and MRS +20% glycerol agar respectively. The plates were then incubated at 37 ^{o}C for 2 days followed by overlaying with the *E.coli* in LB+ 0.7% soft agar and the overlaid plates were incubated at 37 ^{o}C for a day.

The candidates that represent the antimicrobial activities would be able to produce clear zone around the colonies. The positive colonies on the MRS plates were

represented as the bacteriocin producing strains whereas the positive colonies on the MRS +20% glycerol were represented as reuterin producing strains.

5. Test of the ability of antimicrobial production suspected to be 3-HPA and bacteriocin production using filter disc method

After collecting the candidates with the antimicrobial activities, the candidates were grown in the 10 ml MRS broth, incubated overnight at 37 ^{o}C for 2 days, then centrifuged at 5000 rpm for 2 minutes .The pellet was kept, the pellets of candidates having the abilities to produce the bacteriocin was resuspend in MRS broth where the pellets of candidates having the possible ability to produce 3 HPA was resuspend in the 80 % v/v glycerol solution and incubated overnight.

After the overnight incubation, the sample were centrifuged again at 5000 rpm for 2 minutes .The supernatant from the candidates were tested on the presence of the antimicrobial agent suspected to be 3-HPA and bacteriocin using filter disc method using 2 fold dilution (0x ,2x,4x,6x). Before testing on the supernatant, the supernatant from each candidate was sterilely filtered using 0.22um Millipore filter to get rid of the cells.

Sterile supernatant of each candidate was then diluted to 0x, 2x, 4x, 6x . Ampicillin was used as the positive control, *Lb reuteri* was used as the positive control and glycerol solution was used as the negative control for 3-HPA determination where for the bacteriocin determination, Ampicillin as the positive control , MRS solution was used as the negative control.

The plates used for the determination of the antimicrobial agent suspected to be 3-HPA production and bacteriocin production contained LB+1.5% soft agar mixed with the *E.coli* of growth of 1.05 x 10^4 Cfu/ml. Each control and sample solution was added in the amount of 20 µl on the paper disc and place on the top of the plate the was spreaded with *E.coli*

6. Standard curve of OD VS Cfu/ml of *E.coli.*

E.coli standard growth curve was constructed in order to know the time when the population of the *E.coli* would be equal to 10^4 CFU/ml because this concentration of the *E.coli* was considered to be the suitable amount for overlaying in the determination of antimicrobial activity. (Land, 1992)

E.coli culture grown on the LB agar was transferred to 5 ml LB broth and incubated overnight. One milliliter of *E.coli* in LB broth was transferred to 250 ml flask containing 50 LB broth for shaking at 180 rpm at room temperature and then OD was measured every 1 hour at 600 nm. The spread plate method was used in this part; the spread plate technique was performed every hour. The dilution for the spread plate technique was preformed by using the 0.1 % peptone, the dilution was varied according to the concentration of the *E.coli* amount, at the earlier time point of the cultivation of *E.coli* , the dilution was performed in lower dilution while at the later time point of the cultivation, the higher dilution was performed .At each dilution dilution ,the spread plate was performed duplicate.

The OD_{600} was measured and spread plating technique was performed every hour until the *E.coli* growth reaches the stationary phrase. All the plates were incubated overnight at 37 °C, and then checked the CFU/ml of each dilution.The standard curve was plot between and $OD_{600}$and CFU/ml.

7. *L. reuteri* culture preparation

L. reuteri was used as the reference strain for the production of 3-HPA. *L. reuteri* was grown on the MRS plate and incubated at 37 °C for 2 days. For the determination of the antimicrobial activity, *Lactobacillus reuteri* on the MRS plate was grown on the 80% glycerol, and than transferred to the pellet to MRS ,incubated overnight, centrifuged at 5000 rpm for 2 minutes and filtered with 0.22um Millipore filter before testing.

8. Ampicillin preparation

Ampicillin at the concentration of 1,230 ug/ml was used as a positive control for the determination of antimicrobial agent suspected to be 3 –HPA. Ampicillin was prepared by dissolving 0.01 g of the ampicillin power in 1 ml of distilled water (master stock) then performed the dilution up to the dilution of 10^{-2} by pipetting 100 µl of ampicillin from the master stock to new tube containing 900 µl of distilled water (10^{-1}) and pipetting another 100 µl of ampicillin to another new tube containing 900 µl of distilled water(10^{-2}) to make the ampicillin concentration of 100µg/ml. Ampicillin was filtered to get rid of the possible cells by using 0.22um Millipore filter.

9. Checking of pH of supernatant

Supernatants that were collected from both determination of antimicrobial agent suspected to be 3-HPA and bateriocin were measured the pH by using the dilution 0X of the supernatants. pH value was measure by using the pH meter.

RESULT AND DISCUSSION

Lactic acid bacteria were isolate from the, fish and piglet intestines from the fresh market in Bangkok. LAB were screened out for the ability to produce 3 HPA and bacteriocin, compared to the positive strain of antimicrobial agent suspected to be 3 HPA production (*L. reuteri*) and positive control of ampicillin. Directed plating method with MRS-$CaCO_3$ agar was initially used for isolation of LAB .Calcium carbonate was function as an indicator to detect the acid producing colonies by having the clear zones around the colonies due to $CaCO_3$ hydrolyzing. One hundred and ten colonies were obtained from chicken and pig intestines then, the colonies were streaked on the MRS+20% glycerol to purify the colonies prior to conducting LAB confirmation.

Figure 3: acid producing bacteria colonies appearing a clear zone on MRS-$CaCO_3$ media

The results showed that 45 isolates from 110 isolates seemed to be LAB based on confirmation test. Forty five isolates of *LAB* were tested for their ability to produce antimicrobial agent suspected to be 3-HPA and bacteriocin as the antimicrobial effect on the growth of the indicator strain *E. Coli.* Five out of forty five isolates were suspected to be 3-HPA producing strains where seven out of forty five isolates were suspected to be bacteriocin producing strains

Inhibition for each isolate was determined by the presence of clear zone around the colonies on the MRS and MRS+20% glycerol for Bacteriocin and antimicrobial agent suspected to be 3-HPA testing respectively. The candidates that represent the ability to produce antimicrobial agent suspected to be 3 HPA were grown on MRS+20% glycerol because the tested candidates could use glycerol as the substrate to convert to 3 HPA. The 9 isolates from 45 isolates showed inhibitory activity against *E. coli* from the overlay method .These 9 strains were selected for further studied for 3-HPA production and were named as F, I, J, L, O, P, R, 10, 25 (Table 3). The strains were isolated mostly from chicken and piglet intestines .All of the candidates isolated from the chicken intestine showed the cocci shape which may be classified in the genera *Enterococcus, Lactococcus ,Streptococcus* and *Vagococcus* where the candidates isolated from the piglet intestine showed the rod shape which may be classified in the genus *Lactobacillus acidophilus, L. bulgaricus* as well as the reference strain.

Table 3: Phenotypic characteristics of the candidates and the reference strain.

Candidates	Phenotypic characteristics					
	Sources	Catalase	Morphology	Gram staining	Spore staining	Motility
F	Chicken intestine	-	Cocci	+	-	-
I	Chicken intestine	-	Cocci	+	-	-
J	Chicken intestine	-	Cocci	+	-	-
L	Chicken intestine	-	Cocci	+	-	-
O	Chicken	-	Cocci	+	-	-

	intestine					
R	Chicken intestine	-	Cocci	+	-	-
P	Chicken intestine	-	Cocci	+	-	-
10	Piglet intestine	-	Rod	+	-	-
25	Piglet intestine	-	Rod	+	-	-
Lb. reuteri		-	Rod	+	-	-

Before performing the antimicrobial determination, the *E.coli* standard growth curve was constructed in order to know the time and optical density when the population of the *E.coli* would be equal to 10^4 CFU/ml because this concentration of *E.coli* was considered to be the suitable amount for overlaying, According to the reference that 3 HPA represented a limitation on the inhibitory activity over the growth of *E. coli*. For the determination of the antimicrobial activity, the isolates having the antimicrobial spectrum will be able to compete with certain concentration of the pathogen which was *E.coli* in this research, If the concentration of the *E.coli* was too high, the clear zone around the colonies would be smaller and difficult to observe or couldn't even see the possible clear zone around the colonies. If the concentration of the *E.coli* was too small, the clear zone that could present around the colonies would be like a lawn which was quite difficult to observe (*SL Buck - 1996*)

At OD_{600} around 0.5 (approximately 4 hours of flask shaking), the concentration of the *E.coli* reached 10^4 CFU/ml, so the *E.coli* was then taken for overlaying in the determination of antimicrobial activity.

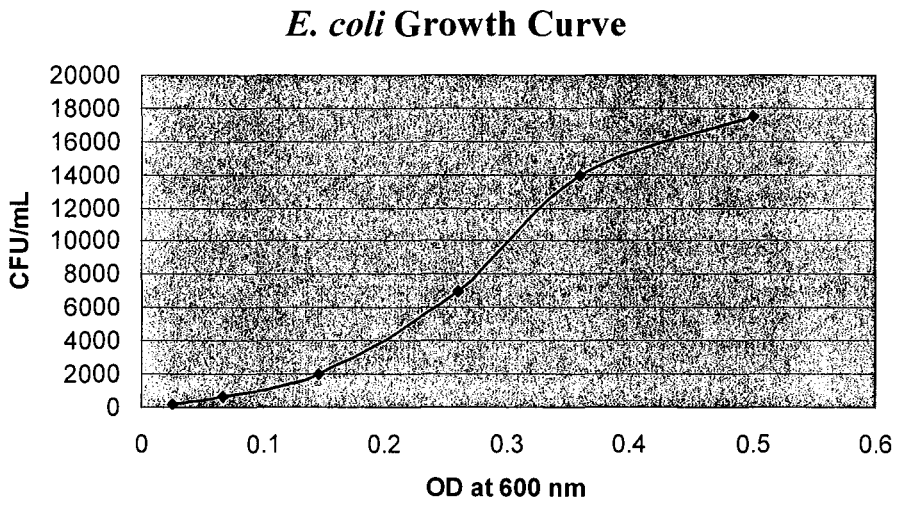
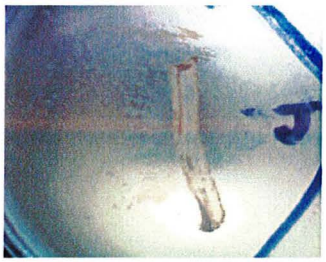


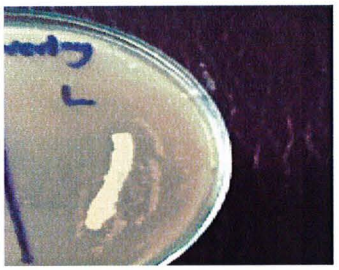
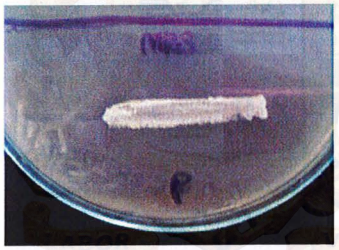
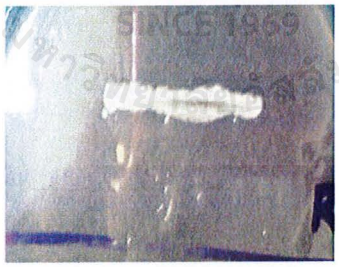
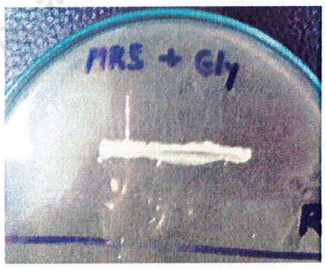
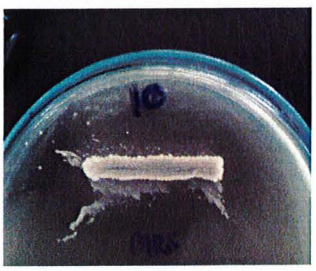
Figure 4: Standard growth curve of *Escherichia coli* ATCC 25922

Nine isolates were confirmed to be the LAB as they were Gram-positive, non-spore forming, rod and cocci, non motile, negative catalase test. As shown in Table1 Most of these characteristics suggested that these isolates could belong to the genus *Lactobacillus* (Axelsson *et al.*, 1998). Then paper disc method was used to determine the production of 3-HPA and bacteriocin.

Moreover, their antimicrobial activity against the indicator strain (*E. coli.*) was investigated. The result showed that all of the candidates displayed the antimicrobial activity by appearing the clear zone around the candidates from the overlay method. Clear zone around the candidates appeared on MRS plate and on MRS+20% glycerol were referred as bacteriocin and antimicrobial agent suspected to be 3-HPA producing strains respectively.

Table 4: Antimicrobial spectrum against the growth of the indicator strain, *E. coli* of each candidate on different agar plate: MRS plate and MRS + 20% glycerol plate

Candidates	Antimicrobial activity against the growth of indicator strain	
	MRS	MRS+20%Glycerol
F	-	
I		
J		-

L	-	
O		-
P		-
R		
10		

25		-

Note ***Antimicrobial activity against the indicator strain, the strains were streaked on MRS agar plates for candidates having the ability to produce acteriocin ,MRS +20% glycerol plate for candidates having the ability to produce 3 HPA, then overlaid with *E. coli* and incubated at 37 ^{o}C for 24 hours.

\+ represents the presence of clear zone around the candidates

\- represents the absence of clear zone around the candidates

Production of antimicrobial agent suspected to be 3-HPA and bacteriocin by the candidates and theirs antimicrobial activity against the indicator strain (*E. coli.*) was investigated with the paper disc method using two fold dilutions. We observed that all candidates displayed the antimicrobial activity. For bacteriocin, candidates named I,J,O,P,R,25,10 showed similar antimicrobial activity to the ampicillin where candidates named F,I,L,R,10 showed the similar activity to the reference strain, The AU/ml of candidates was ranged in 200 -300 AU/ml. *Lactobacilus reuteri* (400-600 AU/ml). *L reuteri* has the ability *to* produce a novel broad-spectrum antibiotic substance via the organism's fermentation of glycerol ,the level of the clear zone in each dilution was not the same. At 6x dilutions seemed to be the dilution that gave the smallest clear zone or no clear zones were present among the 9 candidates .while the clear zones were observable among the 9 candidates under the non diluted condition. From the table 2, some of the candidates had broad-spectrum antimicrobial activity by producing both 3-HPA and bacteriocin, so we could observe the clear zone around those candidates for MRS plate and MRS+20%Glycerol plate. Those candidates that had the ability to produce both 3-HPA and bacteriocin were candidates named I, R and 10 where the candidates named F, L had the ability to produce only 3 –HPA and candidates named J, O, P, 25 had the ability to produce just only the bacteriocin.

Table 5: Antimicrobial activity of the candidates having the ability to produce the bacteriocin against the indicator strain at each dilution

Candidates	Bacteriocin				Inhibitory activity of 3- HPA (AU/mL)
	Two fold dilutions				
	0X	2X	4X	6X	
F	-	-	-	-	-
I	++	++	+	+	300
J	++	+	+	-	200
L	-	-	-	-	-
O	++	+	+	-	200
P	+++	++	+	+	300
R	+	+	+	+	300
10	++	+	+	-	200
25	++	++	++	+	300

Note ** Antimicrobial activity of candidates against the indicator strain (*E. coli.*), the strains were cultured in MRS broth and incubated overnight (at 37 °C, 48 h). The inhibitory activity of bacteriocin in cell-free supernatants of MRS solution was determined by the paper disc method. Antimicrobial activity was expressed in arbitrary units (AU) per ml of the original cultures.

AU/ml is the highest dilution that exhibits the clear zone per 1 ml of the supernatant

+++ represents the largest diameter present around the candidates

++ represents the larger diameter present around the candidates

\+ represents the presence of the clear zone around the candidates

\- represents the absence of the clear zone around the candidates

Candidates named F and L showed no clear zone around the candidates due to the reason that they lacked of the ability to produce bacterion that is why there was no clear zone present around the candidates of any dilution. The pH range of the supernatant in the processs of bacteriocin production was around pH 4-5. Therefore, the ability of *E. coli* growth on LB media at pH 4-5 was tested in order to confirm that the effect of the supernatant over the growth of *E. coli* did cause by the low pH of the supernatant.

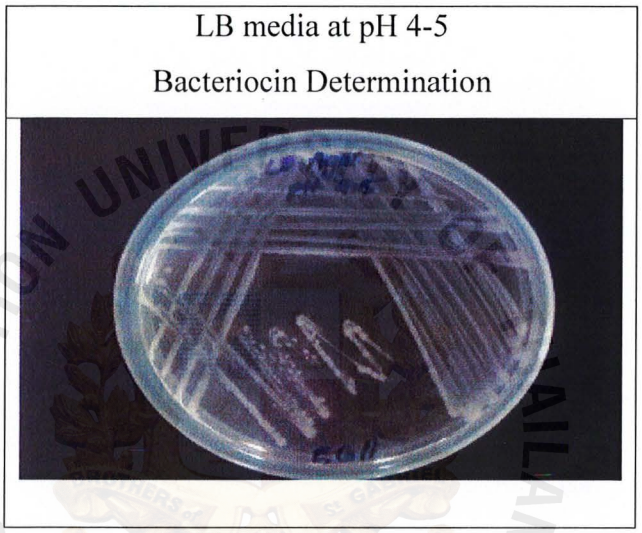


Figure 5: Growth of the *E.coli* on the LB media of pH 4-5 for bacteriocin determination.

The pH of the cell free supernatant was measured, we found that the pH from cell free MRS solution was about 4-5 pH .The result showed that *E. coli* could still grow in the LB media of the pH in the range of 4-5. It indicated that the clear zone present around the candidates were not from the effect of the acidity but from the effect of bacteriocin

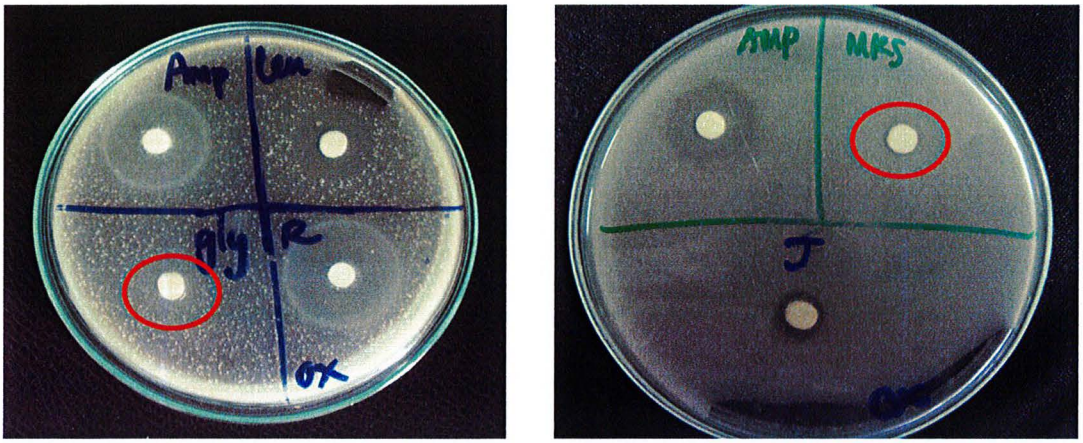
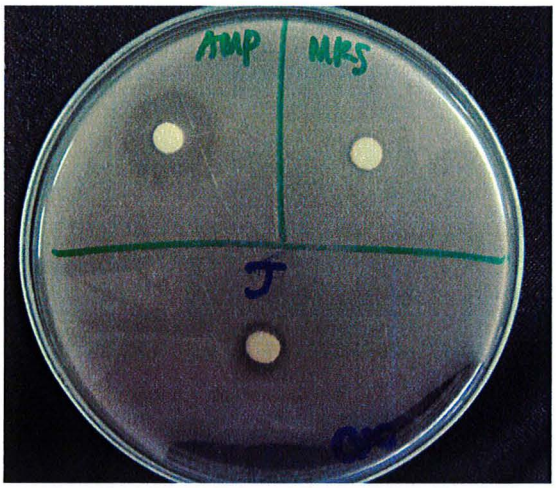


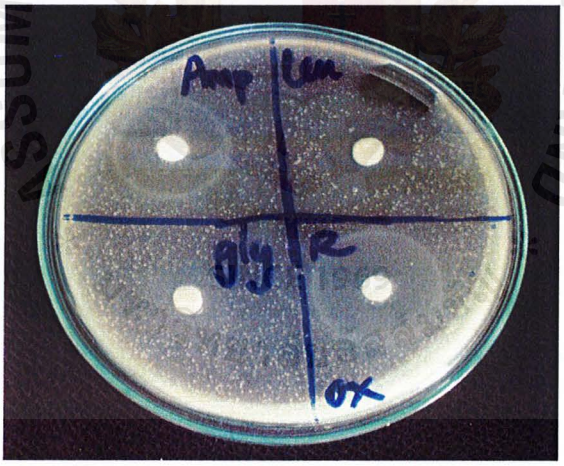
Figure 6: antimicrobial activity of the negative control for the bacteriocin production and antimicrobial agent suspected to be 3-HPA production

For the production of antimicrobial agent suspected to be 3-HPA, 80% glycerol solution was used as the negative control where the ampicillin and the supernatant from the cultivation of *L. reuteri* were used as the positive control. After antimicrobial activity testing, Ampicillin, the supernatant from *L. reuteri* and candidates showed the clear zone around the paper disc but not with the paper disc dropped with 20 micro litters of 80% glycerol solution which function as a negative control. As for the bacteriocin production, MRS was used as the negative control where ampicillin was used as the positive control, after the determination, only ampicillin and candidates showed the clear zone around the paper disc but not around the paper dropped with 20 µl of MRS.



Bacteriocin Production

Figure 7: Antimicrobial activity of the candidates producing bacteriocin at 0x dilution



3-HPA production

Figure 8: Antimicrobial activity of the candidates producing 3-HPA at 0X dilution

Table 6: Antimicrobial activity of the candidates having the ability to produce the 3 HPA against the indicator strain at each dilution

Candidates	3-HPA				Inhibitory activity of 3- HPA (AU/mL)
	Two fold dilutions				
	0X	2X	4X	6X	
F	++	+	+	-	200
I	+	+	+	-	200
J	-	-	-	-	-
L	++	+	+	+	300
O	-	-	-	-	-
P	-	-	-	-	-
R	+++	+	+	+	300
10	++	++	++	+	300
25	-	-	-	-	-

Note ** Antimicrobial activity of candidates against the indicator strain (*E. coli.*), the strains were cultured MRS broth and MRS broth overnight (at 37 °C, 48 h). The inhibitory activity of 3-HPA in cell-free supernatants of glycerol solution was determined by the paper disc method. Antimicrobial activity was expressed in arbitrary units (AU) per ml of the original cultures.

AU/ml is the highest dilution that exhibits the clear zone per 1 ml of the supernatant

+++ represents the largest diameter present around the candidates

++ represents the larger diameter present around the candidates

+ represents the presence of the clear zone around the candidates

- represents the absence of the clear zone around the candidates

Candidates named J,O,P,25 showed no clear zone around the candidates grown on the MRS+ 20% glycerol plate because they lacked of the ability to covert the glycerol to the 3-HPA substance so there was no clear zone present around the candidates at any dilution where other candidates showed clear zone due to the their antimicrobial activity.

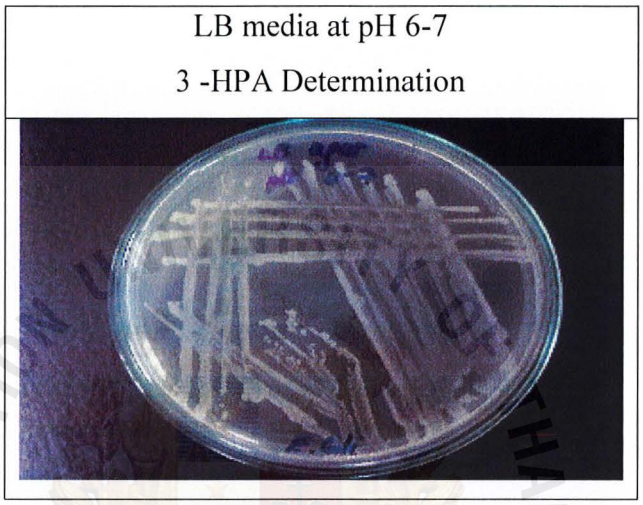


Figure 9: Growth of the *E.coli* on the LB media of pH 6-7 for antimicrobial compound suspected to produce 3-HPA determination.

The pH of the cell free 3-HPA solution was measured in order to know the pH of the solution and adjust the pH to use as the medium for growing the suspected candidates. The pH from the cell free 80% glycerol solution was about 6-7. The result showed that *E.coli* could still grow in the LB media of the adjusted pH which was around 6-7 It indicated that the clear zone present around the candidates were not from the effect of the acidity but from the effect of antimicrobial compound.

Moreover, we measured the diameter of the clear zone around paper disc the, ampicillin and *reuterin* for the determination of the 3-HPA production, it was observed that the diameter of the ampicillin occupied larger diameter than that of the *reuterin* .The negative control (80% glycerol solution0 did not represent any clear zone due to its lack of the antimicrobial activity. For the determination of the bacteriocin, it was observed that ampicillin also produced the lager diameter of the

clear zone than that of the potential candidates where negative control (MRS solution) did not represent any clear zone due to its lack the antimicrobial activity as well.

Table 7: Diameter of the clear zone around the candidate at the 0X dilution of the candidates having the ability to produce bacteriocin.

Candidates	Diameter (mm)	Diameter (mm)	Diameter (mm)
	Candidates	Ampicillin	MRS Negative control
F	-	21	-
I	8	23	-
J	7.5	22	-
L	-	23	-
O	9	25	-
P	8.5	25	-
R	7	22	-
10	8	23	-
25	10	25	-

From table 7, candidates named F and L did not display the clear zone around the candidates due to the lack of the ability to produce bacteriocin where the other candidates displayed the diameter around the candidates. And we could also observe that the clear zone of the ampicillin was somewhat lager than that of the clear zone produced by the candidates because of the higher efficiency of antimicrobial activity comparing with bacteriocin.

Table 8: Diameter of the clear zone around the candidate at the 0X dilution of the candidates having the ability to produce suspected 3- HPA.

Candidates	Diameter (mm)	Diameter (mm)	Diameter (mm)	Diameter (mm)
	Candidates	Reuterin	Ampicillin	80%Glycerol (Negative control)
F	7.5	9	22	-
I	10	8	23	-
J	-	10	19	-
L	7	8.5	20	-
O	-	7	22	-
P	-	8	23	-
R	9	6	22	-
10	7	8	21	-
25	-	9	20	-

From table 8, candidates named J, O, P, 25 did not display the clear zone around the candidates because they lacked of the ability to produce suspected 3-HPA unlike the others. The diameter of the clear zone of ampicillin was larger than that of reuterin and candidates respectively. The diameter of the ampicillin was the larger than that of the reuterin and candidates because of its higher efficiency of antimicrobial activity followed by reuterin and candidates.

These results also support the idea that glycerol as a substrate for production of antimicrobial compound suspected to produce 3-HPA which can be oxidized to acrylic acid, by. For the production of the antimicrobial substance, the candidates susceptible to produce antimicrobial substance were grown in the 80% glycerol

solution merely. This enabled the candidates to use the glycerol as the substrate to produce 3 HPA.

pH from the cell free 80% glycerol solution was about 6-7. The result showed that *E.coli* could still grow in the LB media of the adjusted pH which was around 6-7 It indicated that the clear zone present around the candidates were not from the effect of the acidity but from the effect of antimicrobial compound

CONCLUSION

3-HPA is an important chemical substance used in the industrial production and also used in food application and the production of the bulk chemicals as well as bacteriocins which are considered to be narrow spectrum antibiotics and used in the some certain disease treatments such as cancer treatment. Therefore the screening for the potential strains producing 3-HPA and bacteriocin were investigated.

The result showed that 9 candidates had the ability to produce bacteriocin and antimicrobial compound suspected to produce 3-HPA and most of the candidates were isolated from the chicken intestines bought from fresh market in Bangkok. All candidates showed the similar morphology and the characteristics of the genus *Lactobacillus* spp.

All nine candidates showed the antimicrobial activity against the growth of *E. coli* by displaying the clear zone around the candidates. It can be concluded that the clear zone present around the candidates was not from the acidity produced from the *LAB* themselves but from the production of antimicrobial substance in the supernatant from the conversion of glycerol. Also with the bacteriocin which represented the antimicrobial activity

SUGGESTIONS

In this research, we screened for the Lactic Aid Bacteria that can be able to produce bacteriocin and antimicrobial compound suspected to produce 3-HPA and we could screen for only 9 candidates that had the ability to produce such the substances. Therefore it would be better if we could screen higher number of the candidates suspected to produce 3-HPA and bacteriocin from various sources aside from the chicken and piglet intestines. Moreover, further identification of the candidates and test for their antimicrobial activity against wider spectrum of the pathogenic organisms would be more beneficial for further research and the application in the some disease treatments (Janeiro, 2002).

In addition, in the step of screening for the candidates antimicrobial compound suspected to produce 3-HPA, we grew the candidates on the MRS+20% glycerol which was not so selective for the screening of antimicrobial compound suspected to produce 3-HPA producing by lactic acid bacteria so it would be more specific if we grew the suspected candidates in 80% glycerol media that enables the candidates to use glycerol as the substrate to produce 3-HPA.

REFERENCES

David R. Caprette, Staining and Interpretation of Smears, *Rice University 2005, p.1*

Dr. Ronald E. Hurlbert, 1998, Microbiology 101 Laboratory Manual, p. 1-2

Elsevier B.V. 3-Hydroxypropionic acid as a nematicidal principle in endophytic fungi. Volume 65, Issue 15, August 2004, Pages 2239-2245

Farkas-Himsley H,, Bacteriocins--are they broad-spectrum antibiotics, *J. Antimicrob. Chemother, 2005,* 6 (4): p. 424–6

Juan Carlos Espinosa, Monica Fernandez-Gonzalez, Juan Ubeda and Ana Briones, Identification of Wine Yeasts by PCR-RFLP without Previous Isolation on Plate, p. 157–160 (2002)

Koneman, Allen, Janda, Schrechenberger and Winn. 1997. Color atlas and textbook of diagnostic Microbiology. p. 404-405

Korakoch Hamsupo, Mallika Boonmee, Piyaporn Udomsri, and Penjan Putongpan, Screening of Lactic Acid Bacteria Producing 3-Hydroxypropionaldehyde from Various Sources, International Conference on Science & Technology: Applications in Industry & Education, 2008 p. 2223-2226

Land. 1992. *In* Isenberg (ed.), Clinical microbiology procedures handbook, vol.1. American Society for Microbiology, Washington, D.C. p. 404-405

Mathews ,Van Holde, and Aherm, Lecture 4 Metabolism, 2008 p.1-10

Ponsuk Prasertsub and Pawadee Methacanon, Isolation Selection and Identification of Microorganism Enable to Produce 1,3 Propanediol from Glycerol Residues of Biodiesel Production, 2008, p 1

Rio de Janeiro, Identification of a criminal by DNA typing in a rape case in, Brazil, vol.120 no.3 May 2002

Todd L. Talarico and Walter J. Dobrogosz, Purification and Characterization of Glycerol Dehydratase from *Lactobacillus reuteri,* Department of microbiology, North Caroliina State University, 1990, p.1

Tomas James Rees, The Development of a Novel Antifungal Silage Inoculant, Cranfield University Biotechnology Centre, UK, 2004, p.1

Appendix A

Media

1 MRS medium + 0.5% w/v $CaCO_3$

MRS agar	31 g
$CaCO_3$	2.5 g
Water	500 ml
Total volume	500 ml

2. MRS medium

MRS agar	31 g
Water	500 ml
Total volume	500 ml

3. MRS broth +20% v/v glycerol

MRS broth	22.06 g
Glycerol	80 ml
Water	400 ml
Total volume	500 ml

4. MRS medium +20% v/v glycerol

MRS agar	24.8 g
Glycerol	80 ml
Water	400 ml
Total volume	500 ml

10. MRS broth + 50% v/v glycerol

MRS broth	2.7575 g
Glycerol	25 ml
Water	50 ml

Total volume	50 ml

11. LB+0.7% soft agar

Peptone	2.5 g
Yeast extract	1.25 g
Sodium chloride	2.5 g
Water	250 ml
Soft agar	1.75 g
Total volume	250 ml

12. LB+1.5% soft agar

Peptone	5 g
Yeast extract	2.5 g
Sodium chloride	5 g
Water	500 ml
Soft agar	7.5 g
Total volume	500 ml

13. LB broth

Peptone	2.5 g
Yeast extract	1.25 g
Sodium chloride	2.5 g
Water	250ml
Total volume	250 ml

14. MRS broth

MRS broth	27.575 g
Water	500 ml
Total volume	500 ml

11. 80 %Glycerol solution

Glycerol	400 ml
Water	100 ml
Total volume	500 ml

Appendix B

E.coli standard growth curve

Time (hr)	OD at 600 nm	Dilution			
		10^{-1}	10^{-2}	10^{-3}	10^{-4}
0	0.026	2	-	-	-
1	0.067	9	5	-	-
2	0.146	TNTC	6	1	-
3	0.260	TNTC	8	4	-
4	0.360	TNTC	TNTC	7	2
5	0.5	TNTC	TNTC	8	3

Hour 0: At dilution 10^{-1}

(2 x 10)/0.1 = 200 cfu/ml = 3.9x 10-3

At dilution10^{-2}

(1 x 100)/0.1 = 1000 cfu/ml

Average Cfu/ml = (200 +1000)/2 = 600Cfu/ml

Hour 1: At dilution 10^{-1}

(9 x 10)/0.1 = 900 cfu/ml

At dilution10^{-2}

(2 x 100)/0.1 = 1450 cfu/ml

Average Cfu/ml = (900 +200)/2 = 1450Cfu/ml

Hour 2: At dilution10^{-2}

(6 x 100)/0.1 = 6000 cfu/ml

At dilution10^{-3}

(1 x 1000)/0.1 = 10000 cfu/ml

Average Cfu/ml = (6000 +10000) /2 = 8000 Cfu/ml

Hour 3 At dilution10^{-2}

(8 x 100)/0.1 = 8000 cfu/ml

At dilution10^{-3}

(4 x 1000)/0.1 = 40000Cfu/ml

Average Cfu/ml = (15000+50000) /2 = 24000 Cfu/ml

Hour 4 At dilution10^{-3}

(7 x 1000)/0.1 = 70000 Cfu/ml

At dilution10^{-4}

(2 x 10000)/0.1 = 20000 Cfu/ml

Average Cfu/ml = (70000 + 20000) /2 = 45000 Cfu/ml

Hour 5 At dilution10^{-3}

(8 x 1000)/0.1 = 80000 Cfu/ml

At dilution10^{-4}

(3 x 10000)/0.1 = 30000 Cfu/ml

Average Cfu/ml = (80000 + 30000) /2 = 55000 Cfu/ml

Appendix C

pH of cell free MRS supernatant (Bacteriocin Testing)

Cell free MRS supernatant	pH value
F	4.03
I	4.25
J	4.78
L	3.97

O	4.56
P	4.45
R	4.56
10	3.89
25	5.06

pH of cell free 80% glycerol supernatant (3 HPA Testing)

Cell free MRS supernatant	pH value
F	6.77
I	7.32
J	6.54
L	6.91
O	7.18
P	7.03
R	6.96
10	7.23
25	6.45