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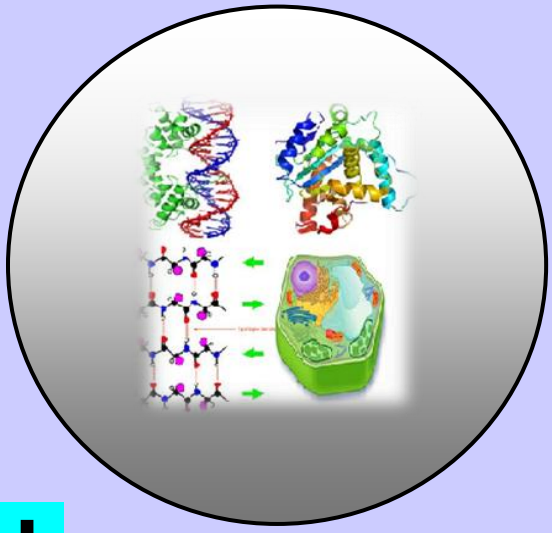

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RESEARCH PAPER
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Examination of Lactic Acid Bacteria's Ability from Fish Intestinal Tract in Deconjugation Bile Salt

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ABSTRACT

The study was carried out to determine the ability of lactic acid bacteria (LAB) isolated from fish intestine to do deconjugation of bile salt. Bile salt deconjugation was shown by the production of cholic acid. This experiment was consisted of six treatments with five replicates. The treatments in thus experiment were addition of medium with bile salt equal to 0, 1.0, 0.2, 0.3, 0.4, and 0.5%. Lactic acid bacteria were grown in medium at 39^{o}C for 20 h. Variables observed were density per h., pH, and cholic acid produced in the end of fermentation process. The data obtained were analyzed using variance analysis with one way of Completely Randomized Design (CRD), differences between mean values of each variable were tested by Duncan's Multiple Range Test (DMRT). The changes of density show that LAB was able to grow in medium that added bile salt until level of 0.5%. Nevertheless, the addition bile salt in the medium decreased the growth rate of LAB during exponential phase. The production of cholic acid were 0.005, 0.087, 0.175, 0.211, 0.202, 0.150 µmol/ml, while the pH values were 4.6, 4.7, 4.92, 5.00, 5.05 and 5.15 respectively for the addition of 0, 0.1, until 0.2% in added 0.5% the production cholic acid decrease significantly.

It could be concluded that LAB isolated from fish intestine was able to do deconjugation of the bile salt until level 0.4%.

(Key Words: Lactic Acid Bacteria, Bile Salt, Deconjugation, Cholic Acid and Fish Intestine.

INTRODUCTION

The increase of public awareness in the health importance nowadays causes people to be more careful in selecting food that they consume. Dairy products especially meat belong to the kinds of nutritious and delicious food. Thus, many people like to consume them. It is proved by the increasing of meat consumption year after year in line with the increase of people's degree in their life.

Cholesterol, on the one side, is needed by our body. Cholesterol is an important component for the body mostly for composing membranes, bile acid, and hormones. On the other hand, excessive amount of cholesterol in the body may cause several health issues. A high level of cholesterol in human food can raise the level of cholesterol in the blood serum which can increase the risk of arteriosclerosis and coroner heart disease (Lubbadeh et.al., 1999). The absorption of cholesterol depends upon the availability of bile acid from the liver, esterase cholesterol enzyme from the pancreas, and also based on the amount of consumed cholesterol (Ensminger et.al., 1995)

There are a lot of works done by scientists to overcome the problem. In the dairy field, it is attempted to provide low cholesterol dairy products. One of the ways to reduce the cholesterol level is done by the use of probiotic. Widodo (2003) explained that one of the familial groups used as probiotic was lactate acid bacteria (LAB).

There are a lot of advantageous LAB roles in the sector of health and nutrition. LAB can increase the digestibility of food nutrients, control the intestinal infections, increase the lactose digestion, control cancers blood cholesterol level (Gilliland, 1990).

In the last few years, it has been advanced the use of lactate acid bacteria to influence the cholesterol level in the human and cattle (Tanaka et al., 1999). The reduction of cholesterol by LAB is assumed to be able to work directly by using cholesterol assimilation and indirectly by using the mechanism of bile salt deconjugation.

The mechanism of bile salt deconjugation is happened indirectly during the enterohepatic cycle or fat absorption cycle from the intestine (Drassar and Barrow, 1995). Without the process of deconjugation, bile salt will be absorbed back into the small intestine.

LAB isolate from the Tawes fish intestines *(Puntius javanicus)* had been acquired in the previous research. That LAB is hoped to have a role as prebiotic which is able to lessen the cholesterol level which in this case will be seen from its ability to do the bile salt deconjugation.

Research Objective

The examination of LAB abilities from the Tawes fish alimentary tract *(Puntius javanicus)* to grow and do the bile salt deconjugation had been done in this research.

Research Significance

The results of this research are expected to be able to be used as information sources for dairy industries in utilizing LAB as probiotics.

Hypothesis

The lactate acid bacteria resulted from the isolation of fish alimentary tract can grow in the medium contained bile acid and do the bile salt deconjugation.

MATERIALS AND METHOD

This research was done in May – November 2005 in the Biochemical Nutrition Laboratory, the Department of Livestock Food and Nutrition, the Faculty of Farms, Gadjah Mada University, Yogyakarta.

Materials

Microbe sources

LAB isolate originated from the Tawes fish intestines; it was taken from the Biochemical Nutrition Laboratory, the Department of Livestock Food and Nutrition, the Faculty of Farms, Gadjah Mada University, Yogyakart.

Chemical substances

The chemical substances used in this research were MRS (Man Ragosa Sharpe), sodium thioglycollate, bile salt (Oxoid brand), NaOH 0,01N, NaOH 1N, HCl 10 N, ethyl acetate 99,5%, NaOH, H_2SO_4 16 N, furfuraldehide 1%, glacial acetate acids, alcohol 70%, gas CO_2,

Tools

The tools used in this research were glasses instruments, *hungate*tube (fermenter), pH meter (Hanna brand), spectrophotometer (Spectronic 21 brand), gas CO_2, *water bath* (Memmert brand), acid cupboard, *centrifuge*, *freezer*, micro pipets, analytic measures, *stirer*, vortex, electrical stove, laminar, sprites lamp, otoklaf.

Methods

Microbes Activation

LAB isolate from stock culture was planted into the MRS broth (Oxoid) medium in the hungate tube (the chemical compositions and the way to make MRS medium are presented in the Appendix 1). The medium volume is 4 ml. The added LAB isolate was 10% from the total medium volume. Gas CO2 was given into the tube to make anaerobe condition. Then 12 hours incubation was done in the temperature of 39^o C. The fermentation result wasa sub-culture which was replanted once more in the same way as before. The fermentation result was used as an activated-culture source.

Fermentation

Active LAB isolate were inoculated 1% into the MRS-thio medium (MRS broth with 0.2%*Sodium thioglycholat*in the *hungate tube*). The medium volume was 12 ml. the bile salt concentration in the medium was controlled from 0%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%. Each treatment was done in 5 repetitions. The degree of acidity in the early fermentation was set at pH 6.5. Then the incubation was conducted in the temperature of 39^{o}C.

Observed Variables

pH value Measurement. The measurement of pH value was done at the end of fermentation in the 20th hour using pH meter (Walker and Gilliland, 1993).

Microbe growth: The observation was done to the density change which was measured using spectrophotometer with the wavelength of 620 nm (Walker and Gilliland, 1993). The observation was done each hour until it got a near-constant density taken from different observations.

Cholic acid level: The examination was done by doing analyses on the cholic acid which had been freed in each culture with Irvin et.al. (1944) method modified by Walker and Gilliland (1993).

Data Analysis

The data taken were analyzed with variance analyses using unidirectional pattern complete random framework continued with the test of Duncan's New Multiple Range Test (DMRT) (Steel and Torrie, 1993).

RESULTS AND DISCUSSION

The Growth of Lactate Acid Bacteria in the Medium of Bile Salt

The pattern of LAB in the medium contained bile salt was measured by observing the drastic change in the medium each hour. The growth of microbes in the liquid medium was shown by the increase of density and the presence of sediment in the bottom of the tube (Gupte, 1990). The average density of LAB growth medium in various concentrations of bile salt is presented on the Table 1.

Table 1. The average density of LAB growth medium in various concentrations of bile salt.

Hour of	0	0.1	0.2	0.3	0.4	0.5
0	0.13 ^{a}	0.14 ^{a}	0.14 ^{a}	0.14 ^{a}	0.13 ^{a}	0.14 ^{a}
0.5	0.16 ^{a}	0.14 ^{a}	0.16 ^{ab}	0.16 ^{ab}	0.13 ^{a}	0.14 ^{a}
1	0.22 ^{b}	0.16 ^{b}	0.19 ^{b}	0.20 ^{b}	0.15 ^{a}	0.14 ^{b}
2	0.44 ^{c}	0.25 ^{c}	0.33 ^{c}	0.35 ^{c}	0.20 ^{b}	0.17 ^{c}
3	0.82 ^{d}	0.51 ^{d}	0.61 ^{d}	0.69 ^{d}	0.36 ^{c}	0.25 ^{d}
4	1.04 ^{e}	0.79 ^{e}	0.81 ^{e}	0.89 ^{e}	0.56 ^{d}	0.62 ^{e}
5	1.22 ^{f}	1.05 ^{f}	0.99 ^{f}	1.05 ^{f}	0.78 ^{e}	0.84 ^{f}
6	1.23 ^{f}	1.12 ^{g}	1.05 ^{g}	1.08 ^{fg}	0.88 ^{f}	0.92 ^{g}
7	1.26 ^{fgh}	1.19 ^{h}	1.08 ^{gh}	1.10 ^{fg}	0.92 ^{g}	0.94 ^{h}
8	1.26 ^{fgh}	1.20 ^{gh}	1.09 ^{gh}	1.12 ^{g}	0.94 ^{gh}	0.97 ^{i}
9	1.26 ^{fgh}	1.21 ^{ij}	1.11 ^{hi}	1.19 ^{h}	0.97 ^{h}	1.04 ^{j}
10	1.26 ^{fgh}	1.23 ^{jk}	1.13 ^{ijk}	1.21 ^{h}	1.03 i	1.09 ^{k}
11	1.27 ^{ghi}	1.23 ^{jk}	1.20 ^{l}	1.32 ^{i}	1.08 ^{j}	1.16 ^{l}
12	1.25 ^{fgh}	1.24 ^{kl}	1.25 ^{m}	1.35 ^{ij}	1.13 ^{k}	1.23 ^{m}
13	1.32 ^{ij}	1.26 ^{lm}	1.34 ^{m}	1.40 ^{j}	1.17 ^{l}	1.30 ^{n}
14	1.30 ^{hij}	1.28 ^{mn}	1.39 ^{m}	1.47 ^{mn}	1.23 ^{m}	1.42 ^{q}
15	1.32 ^{ij}	1.30 ^{no}	1.40 ^{m}	1.53 ^{o}	1.28 ^{n}	1.54 ^{s}
16	1.33 ^{j}	1.31 ^{op}	1.42 ^{m}	1.52 ^{no}	1.40 ^{o}	1.63 ^{k}
17	1.34 ^{j}	1.31 ^{op}	1.40 ^{m}	1.49 ^{no}	1.49 ^{pq}	1.58 ^{p}
18	1.35 ^{j}	1.33 ^{p}	1.40 ^{m}	1.47 ^{lmn}	1.51 ^{q}	1.50 ^{o}
19	1.35 ^{j}	1.33 ^{p}	1.41 ^{m}	1.42 ^{klm}	1.46 ^{p}	1.46 ^{n}
20	1.35 ^{j}	1.30 ^{p}	1.39 ^{m}	1.41 ^{kl}	1.38 ^{o}	1.44 ^{d}

a, b, c, d, e, and f Different superscript in the same column are real different (p<0,05)

On the Table 1, it shows that there are increases in the density during the fermentation at all treatments. It showed that LAB could grow in the medium contained bile salt. The growth was marked by the increase of density for all treatments. LAB still could grow in the medium which had 0.5 % bile salt level. Data on the Table 1 shows that there was LAB growth in the medium contained bile salt. It is shown by the increase of density in the same way with the increase of incubation time. The density increases insignificantly until the 2ndhour, then grows increasing significantly (P<0.05) until the 20thhour.It shows that there was LAB growth phases, according to Hardjo et.al. (1988) that the pattern of microbes' growth consists of 3 phases; they are lag phase which is often called as adaptation phase because the microbes adapt with their environment, in this case is the medium in which the lactate acid bacteria grow. The second phase is exponential phase which is marked by the fast growth of cells and fermentation products. The third phase is the stationer phase which is marked by the static cell growth or as the point in which the growth starts to decline along with its primary product.

The adaptation phase occurred until the 2nd hour and followed by the exponential phase until the 20thhour. Here, the stationer phase was unseen at the 20thhour and there was a reduction of density caused by the more declining amount of living LAB.

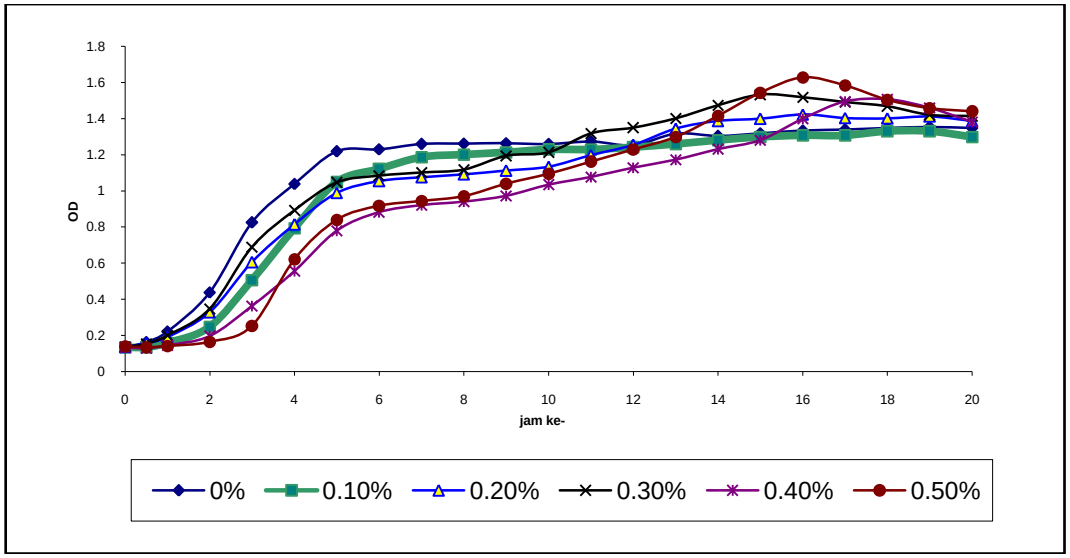

Figure 1. LAB growth graphic in the medium of MRS with the addition of bile salt.

According to the findings, it can be seen that the average lag phase was only occurred until the first half hour. The lag phase was only happened quickly because the medium used in activation was the same with the medium used in the growth medium. The MRS medium nutrient level can be seen in the Appendix. The average exponential phase started to occur on the 1sthour.After the exponential phase, the microbes entered the stationer phase. LAB growth pattern can be seen in the Figure 8 here. The increase of bile salt concentration in the LAB growth medium caused the average density to be lesser significantly ($P<0.01$). According to Dune (2001) that the bile acid shows antibacterial activities which halt the growth of *Escherichia coli* strain, *Klebsiella* sp, and *Enterococcus* sp by in vitro. According to Brandt et.al. (1976) in Oh et.al. (2000) the bile acid shows a retardation effect towards the growth of microbes and this retardation activity is greater than the other organic acids.

According to Bezkorovainy (2001) that the probiotic resistance towards bile salt by in vitro can be divided into two types, they are survival and growth. The survival variance depends on the concentration and the interaction length between the microbes and the bile salt. Other research about the growth of microbes in the medium contained bile salt connect to another variable that is the presence of unconjugated bile acid in the medium. The presence of unconjugated bile acid is the better bacterial lysing agent compared to the conjugated bile acid. The deconjugation of bile salt is done by the bile salt hydrolase enzyme. This enzyme is also produced by Lactobacilli and Bividobacteria. Thus, the increase of bile salt concentration could increase the unconjugated bile acid that could halt the LAB growth.

The presence of bile salt in the LAB growth medium was only as inhibitor. The LAB in the bile salt concentration of 0.3% still showed the growth although it was really different compared to the control (without bile salt).

According to Table 1, there is a difference in occurrence between examinations. The exponential phase for bile salt level examination consecutively 0%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% occurred at the hour of: 1-5, 1-7, 1-6, 1-14, 2-16, 1-15. The exponential phase tended to occur longer in the same way as the higher bile salt level. However if the growth slope was observed, the higher the level of bile salt in the medium, the lower was its slope (figure 2).

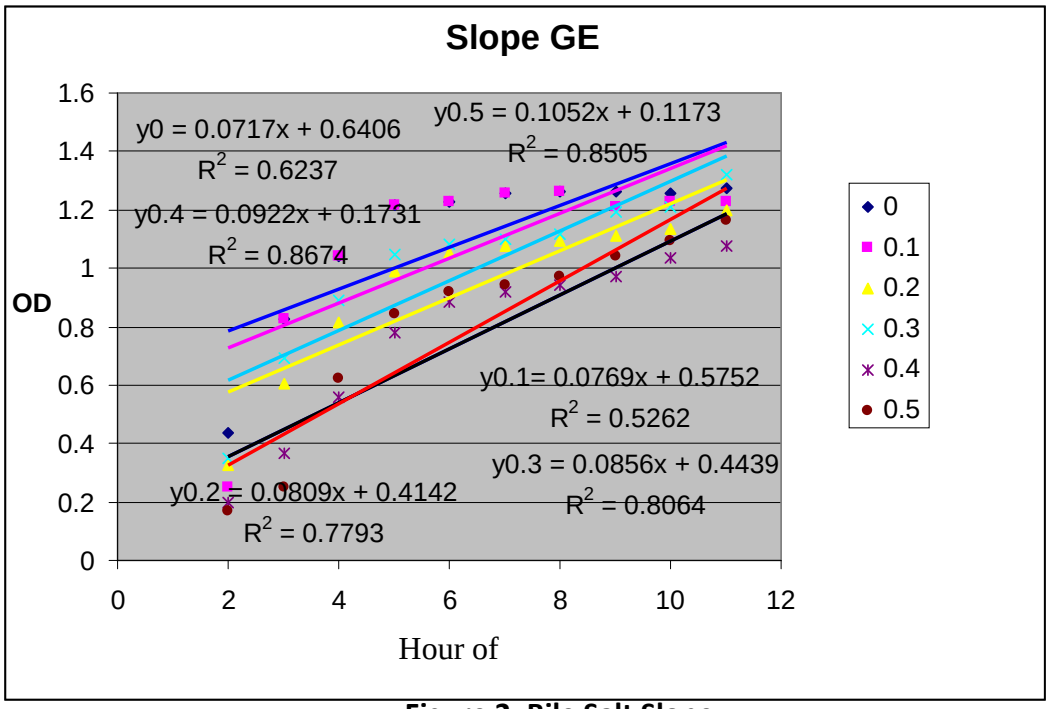

Figure 2. Bile Salt Slope.

The lower of the slope showed that there was a growth obstacle in the medium contained bile salt. Noh et al (2000) explained that the bile salt shows the retardation effects towards the growth of microbes and the retardation activity is bigger than the other organic acids.

The Level of Acidity

pH measurement was done at the 20thhourin order to see the acid products resulted by LAB AST 6. The average pH scores of the medium in the various bile salt are presented in the Table 2.

In the Table 2, until the 20thhour there is occurred a decline in the medium pH from the starting pH 6.5. The decline in pH value during the fermentation time was resulted by the accumulation of fermentation products; they were lactate acid and other organic acids such as acetate and propionate acid. Those organic acids are the final results of glucose hydrolysis by LAB (Mc Donald, 1991).

Table 2. The pH value of LAB fermentation in the medium contained various bile salt at the 20thhour.

Repetition	Bile Salt Level					
	0%	0,1%	0,2%	0,3%	0,4%	0,5%
1	4,64	4,75	4,94	5,01	5,04	5,13
2	4,59	4,72	4,93	5,00	5,06	5,17
3	4,6	4,74	4,90	4,99	5,07	5,15
4	4,59	4,75	4,91	5,01	5,06	5,15
5	4,59	4,74	4,91	5,01	5,04	5,17
Average	4,60^{a}	4,74^{b}	4,92^{c}	5,00^{d}	5,05^{e}	5,15^{f}
± sd	0,022	0,012	0,016	0,09	0,013	0,016

^{a,b,c,d,e,f} **Different superscript in the different column shows a very real difference (P<0.01)**

The research findings showed that the bile salt gave a real influence (P<0.01) to the pH in the final fermentation. The level of acidity in the final fermentation was higher and directly proportional with the increase of bile salt concentration in the medium.

The difference in average value of pH related to the retardation effect of bile salt to the growth of LAB. The retardation to the growth caused the LAB abilities to decline in producing the final product which was in the form of organic acid especially lactate acid. Organic acids resulted during the fermentation were very determining the decline of pH during fermentation. At the higher concentration of bile salt, the pH of final fermentation was also higher. The retardation effects of bile salt to the growth of LAB resulted in the decline of LAB abilities in producing final products which was in the form of organic acids especially lactate acid. The presences of these organic acids were very influencing to the decline of the medium's pH. With the increase of bile salt concentration in the growth medium caused an increase to the retardation effect to the LAB growth (Table 1). It can also be seen in Table 2, the increase of bile salt concentration follows by the increase of average medium's pH. Although there were growth retardation activities by bile salt, LAB was still able to survive and grow in the medium contained bile salt until the concentration of 0.3%. It could be seen by the increase density (Table 5) and the lesser pH (Table 2) at the final observation time, although showed a real difference compared to the control (without bile salt). At the end of observation, the average medium's pH could reach critical pH that is about 4. At this critical pH, the pathogenic microbes could not survive (Gilliland, 1990). According to Pereira and Gibson (2002) that the survival ability and growth parameter at the pH of 3.0 during 2 hours and grows in the medium contained 1.000 mg/1 bile used as consideration standard of the culture microbe's survival abilities towards the acids and bile.

According to Jack et.al. (1995); Montville and Kaiser (1993) in Oh et.al. (2002) that LAB produces some antimicrobial compound such as organic acids (lactate, acetate, propionate, and format acid). The LAB's abilities in producing these organic acids were reflected to the decline of medium's pH, on the 20thhour the medium's pH could reach 4, 74 (Table 2) and at the bile salt concentration of 0.5 %, LAB was still able to decline the pH until 5.15.

Bile Salt Deconjugation

The examination results of cholic acid production at the end of fermentation with various levels of bile salt are presented on the Table 3.

Table 3. Collate acid produced from the result of bile salt deconjugation by LAB (micromole/ml).

Replication	Bile Salt Level					
	0 %	0,1 %	0,2 %	0,3 %	0,4 %	0,5 %
1	-0,005	0,078	0,170	0,206	0,184	0,112
2	-0,004	0,096	0,129	0,232	0,227	0,142
3	-0,005	0,065	0,196	0,196	0,167	0,144
4	-0,005	0,089	0,172	0,201	0,206	0,117
5	-0,008	0,105	0,210	0,220	0,226	0,233
Average	-0,005^{d}	0,087^{c}	0,175^{ab}	0,211^{a}	0,202^{a}	0,150^{b}
± sd	0,002	0,015	0,030	0,015	0,026	0,049

^{a,b,c,d} Different superscripts in the same row show a very real presence of difference (P<0,01).

Lactate acid bacteria which could do the bile salt deconjugation are bacteria which can produce bile salt hydrolase enzyme. The bile salt hydrolase enzyme will hydrolyze the glycolic acid and taurocholic acid from the bile salt to form cholic acid and cenodeocicolate acid. Cholic acid has no role in fat emulsion and does not absorb back in the intestine in the enterohephatic cycle. Cholic acid will be thrown out along with the feces (Drassar and Barraw, 1995).

The results of statistical analysis showed that the cholic acid produced was really increasing ($P<0.01$) at the addition of bile salt 0.1% until 0.5%. These results showed that the observed LAB isolate had the ability to do bile salt deconjugation.

Freed cholic acid was increasing in a very real way ($P<0.01$) at the addition of bile salt until 0.2%, but the bile salt level between 0.2% until 0.4% showed no real changes. At the addition of 0.5% bile salt, the freed cholic acid decline in a very real way ($P<0.01\%$) lower than the addition of bile salt 0.3%. This result showed that the observed LAB had the abilities to do bile salt deconjugation maximally at the level of 0.2%. Although at the level of 0.4% the deconjugation activities were still detected. At the concentration level of 0.5% bile salt, the LAB's abilities to do deconjugation would lesser. The decline in abilities to do deconjugation in this case related to the decline of LAB's growth as a process resulted from the retardation by bile salt.

The LAB's abilities to do this bile salt deconjugation showed that LAB AST 6 had a potency to be probiotics which was able to lessen the cholesterol level. As it had explained by Tanaka (1999) that the ability to do bile salt deconjugation is the main mechanism in lowering cholesterol level. The reduction of cholesterol level resulted from the bile salt deconjugation is occurred in the alimentary tract within the body. In the alimentary tract, it is occurred the digestive reduction of food cholesterol, because in digesting the cholesterol, bile salt is needed to role as emulsion (Guyton, 1974). The lessening availability of bile salt to help cholesterol digestion causes the cholesterol digestion to decline. Undigested cholesterol will be thrown away along with the feces (Center, 1993 cited by Lubbadeh, 1999).

Lost bile salt after deconjugation in the alimentary tract and being thrown away along with the feces will be replaced by new bile salt synthesized in the lever. Synthesized bile salt in the lever takes cholesterol tissue as the basic materials (Volwiler et.al. 2006). Through that process, the cholesterol level in the body can decline.

CONCLUSION AND SUGGESTION

Conclusion

From the research findings, it can be concluded that isolated LAB from the alimentary tract are able to do bile salt deconjugation. The high deconjugation happened at the bile salt level of 0.2%. The deconjugation still occurred well at the bile salt level of 0.4%.

Suggestion

This research should be continued with the examination of LAB's abilities to be able to grow and attach on the wall of the alimentary tract as one of the requirements for LAB to be able to role as probiotics.

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