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Screening test of Bile Salt Hydrolase activity produced by *Lactobacillus* spp.by qualitative and quantitative methods

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ABSTRACT

Bile salt hydrolase (BSH) activity of intestinal bacteria including (*lactobacilli*) is one of the indirect ways of decreasing a cholesterol level in human body. Tested *Lactobacillus* strains were isolated from faeces of fully breast-fed infants, identified by phenotypic methods and biochemical tests screening for production BSH by direct plate assay method, and quantitative method.Among12 lactobacillus isolates ,only 3 strains were shown to be positive for BSH activity by agar plates supplemented with 0.5 % (w/v) Na-taurocholate (TCA) and 0.37 g/L of $CaCl_2$. While 10 isolates showed activity in a quantitative assay. The results shown quantitative assay was more sensitive compared with the plate assay (qualitative method) for BSH activity determination.

Keywords: *Lactobacillus*, BSH, bile salt hydrolase activity.

1. INTRODUCTION

Bile salt hydrolase (E.C.3.5.1.24) is an enzyme which plays a significant part in bile acid metabolism. That found in many bacteria constituting the mammalian gut microbiota, this enzyme catalyzes the hydrolysis of taurine and glycine-conjugated bile acids into free bile acids and amino acid residues [1] which are less reabsorbed than their conjugated counterpart. This result in the excretion of larger amounts of free bile acids in feces. Therefore, deconjugation of bile salt lowers serum cholesterol by increasing the demand for cholesterol for de novo synthesis of bile acids to replace those driven out in feces. The amount of bile salts that returns to the liver is decreased, resulting in a loss of (Feedback inhibition) of bile salt synthesis and increased conversion cholesterol to bile salts [2]. BSH enzymes are represented in various microbial species across most phyla. With a focus on commensal gut microbes BSH activity has been reported in *Lactobacillus* [3, 4]. *Lactobacillus* bacteria contribute 86 and 74% of total bile acid hydrolase activity in the ileum and cecum [5]. Although the activity of BSH is low or not detected in those bacteria that are not commensal in the GIT, the *bsh* gene can be acquired horizontally among different micro-organisms. BSH enzyme gives an advantage to these micro-organisms to survive and colonize the small intestine since the conjugated bile salts have an anti-bacterial effect [6]. BSHs act on a wide range of bile acid conjugates, including all six major human conjugated bile acids glycodeoxycholic acid (GDCA), glycocholic acid (GCA), taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA) and taurodeoxycholic acid (TDCA) releasing glycine- or taurine and free bile salts [7, 8] making it one of the most bacteria that play a major role in bile salts hydrolysis *in vivo*. Deconjugation of bile salts by BSH may encourage antagonist action of some intestinal flora such as *lactobacilli* against pathogens in the intestines; it also results in the formation of more toxic compound such as deoxycholic acid and cholic acid. BSH-active strains formed a type of self-defense mechanism versus these toxic compounds [9].

2. MATERIALS AND METHODS

2.1 Isolation and Identification of *Lactobacillus* isolates

A total of 40 stool samples were collected in the sterilized screw capped from fully breast-fed infants. The samples received were streaked on MRS agar plates .Then all plates were incubated anaerobically with gas pack at 37°C for 48hrs. Then, small with entire margin, convex, smooth, glistening, and opaque without pigment colonies, recultured on MRS agar for the identification at genus level by sugar fermentation test using (Glucose, Maltose, Mannitol ,Mannose, Sucrose, Ribose, Raffinose Fructose, Galactose ,Xylose, Rhamnose and Arabinose) and cultural tests included, motility test, oxidase test, catalase, arginine hydrolysis test, gelatinase test and growth at 6.5% NaCl as well as 2% bile salt . The highest BSH production isolates re-identified at species level was accomplished by 16S-ribotyping and verified by sequencing of representative PCR products at Macrogen (Seoul,Korea). Genomic DNA was extracted from bacterial cultures with G- spin DNA extraction kit (intron biotechnology/Korea) according to manufacturer's instructions. The universal primer Forward 27F 5′-AGAGTTTGATCCTGGCTCAG3′ Reverse1492R 5′- GGTTACCTTGTTACGACTT-3′ was used for 16S rRNA gene amplification [10].

2.2 Detection of bile salt hydrolase

2.2.1 Direct plate assay (qualitative assay)

All tested bacteria (12 isolates) subculture in MRS broth and incubated anaerobically in an anaerobic gas pack jar at 37°C for 24 h. sterile filter disks paper were impregnated in an overnight culture of the test isolates and placed on MRS agar plates supplemented with 0.5% (wt/vol) taurocholic acid and 0.37g/L of CaCl2. The plates were incubated anaerobically at 37°C for 72h. MRS agar plates without bile salt supplementation were used as control. The presence of precipitated bile acid (cholic acid) around colonies (opaque halo) or the formation of opaque granular white colonies was considered as a positive reaction [11].

2.2.2 Quantitative assay for bile salt hydrolase activity

BSH activity was measured by determining the amount of amino acids liberated from conjugated bile salts by lactobacilli strains as described by Tanaka *et al* (2000) [12]. The concentration of amino acids released was calculated from a standard curve of absorbance at 570 nm derived using glycine briefly, all bacterial isolate cells grown in MRS broth for 24h. Cells were harvested by centrifugation at 8000 rpm for 20 minutes. The supernatant was discarding and the pellet was washed twice with 0.1M sodium phosphate buffer pH 6.5 containing 20mM α- mercaptoethanol and 1mM EDTA. The cell suspension was sonicated for 5 min with constant (cooling ice) (1/2 min on and 1/2min off), followed by centrifugation at 8000rpm at 4°C for 30 min. The supernatant was collected (crude enzyme). The enzyme activity and protein concentration by (Bradford method) was determined [13].

2.3 Enzyme activity assay

BSH activity was determined with a two-step procedure as following:

2.3.1 First stage

4mM sodium taurocholate (was used as a substrates for BSH) incubated at 37°C for 5 min in water bath. then, 80µl from 0.1M sodium phosphate buffer pH 6.5 (containing 20mM α- mercaptoethanol and 1mM EDTA) was mixed with 20µl of crude enzyme which then added to the substrate, the reaction was incubated at 37 °C for 30 min. The enzymatic reaction was terminated by adding 50µl 15% trichloroacetic acid (TCA).The precipitated protein was removed by microfuge with high speed for 15min. The supernatant was taken for BSH activity assay determination.

2.3.2 Second stage

20µl from supernatant was mixed with 80µl distilled water in test tube and 1.9ml ninhydrin reagent added to each tube, the reaction mixture was incubated in a boiled water bath for 15 min. The absorbance of the cooled sample was measured at wavelength at 570 nm. BSH activity (unit /ml):- was defined as the amount of enzyme that released 1µmol of taurine /glycine from the substrate per minute.

3. RESULTS AND DISCUSSION

All the bacterial isolates were catalase, oxidase and motility negative, and able to grow in the presence of 6.5% NaCl and bile salt. In this study, there was variation in sugar fermentation pattern observed among the isolates. The main task of carbohydrate fermentation test is to investigate the ability of bacteria to ferment different types of sugar and phenol red was used as an indicator to differentiate the bacteria according to their patterns of carbohydrate utilization.

3.1 Qualitative assay of bile salts hydrolases activity

Twelve isolates was screened for their production of BSH, and only three isolate exhibited BSH activity detected by agar plates supplemented with 0.5 % (w/v) Na-taurocholate (TCA) and 0.37 g/L of $CaCl_2$.The activity detected by precipitation zones (cholic acid) around the filter paper on plate the diameter of zones was (16, 14, and 11 mm), respectively as shown in table and figure (1).

Figure 1: Bile Salt Hydrolase (BSH) activity in Lactobacillus isolate in MRS agar supplemented with 0.5% TCA.

Table 1: Most efficient bacterial isolate for bile salt hydrolase production

Isolate code	Precipitation zone (mm)
Lb6	16
Lb8	14
Lb7	11

3.2 Quantitative assay of bile salts hydrolases activity

After the qualitative assay all *Lactobacillus* isolates was examined for their production of BSH by quantitative method (ninhydrin method). Except two, all *Lactobacillus* isolates used in this study displayed a different level of deconjugation activity of TCA (1.21 – 17.25) U/ml table (2). **Lb8** and **Lb6** isolates were showed high level of BSH production was selected for further molecular identification.

Table 2: Bile salt hydrolase activity of *Lactobacillus* isolates.

Isolate code	Enzyme activity (unit/ml)	Protein Conc. (mg/ml)	Specific activity (unit/mg protein)
Lb1	2.22	1.83	1.21
Lb2	–	–	–
Lb3	–	–	–
Lb4	4.06	1.01	4.01
Lb5	3.49	1.03	3.38
Lb6	9.20	1.07	8.59
Lb7	8.57	1.06	8.08
Lb8	17.77	1.03	17.25
Lb9	2.22	1.02	2.17
Lb10	2.53	1.06	2.38
Lb11	4.76	1.12	4.25
Lb12	3.80	1.09	3.48

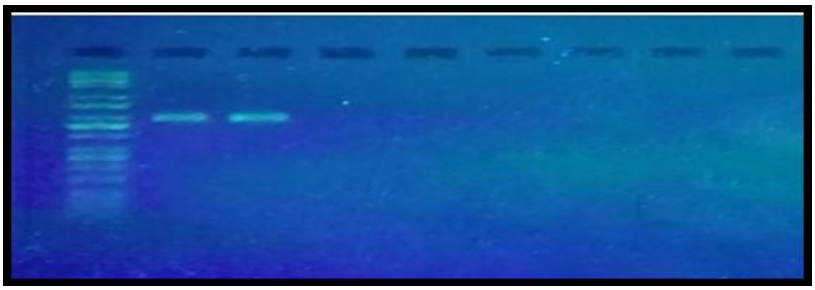

Figure 2: PCR product the band size 1200 bp. The product was electrophoresed on 2% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).

3.3 PCR amplification and sequencing

The 16S rRNA Gene was success fully amplified from both Lb6 and Lb8 isolates using universal 16S rRNA bacterial primers figure (2).

After sequencing results analysis using blast website, acquired outcomes allowed to determine *lactobacillus* at species level. Result indicates lactobacillus Lb8 strains have 97% homology with *Lactobacillus fermentum.* While Lb6 have 98% homology with *Lactobacillus paracasi.*

BSH activity over different bile salts depends on several factors, such as pKa values, pH and polar complementarity toward conjugated bile acids was also considered as an important facet of substrate specificity [14]. Bile salt hydrolase also sensitive to oxygen, strongly inhibited by substances known as sulfhydryl-enzyme inhibitors although addition of reducing agents such as dithiothreitol (DTT) and EDTA might be used to reduce enzyme oxidation and keeping the SH-group of Cys2 in BSH active site under reducing conditions to guarantee the native protein form [12]. Taurine conjugates exhibit an evident pKa of 1.9 in aqueous solution, whereas the unconjugated part the pKa approximately of 5.0. Whereas, Glycine-conjugated bile acids exhibit pKa values approximately of 3.9 thus, could be partially precipitated even without hydrolysis, at pH values achievable by acidic fermentative metabolism, unconjugated form would be protonated and precipitate in the medium, while taurine conjugates would remain completely ionized and remain in solution [15].

However, most BSHs are more efficient at hydrolyzing glycol-conjugated bile salts more than, tauro-conjugated bile salts since glycine-conjugated bile salts are very toxic to bacterial cells at low pH in the intestine, while taurine-conjugated bile salts appear to be less toxic. Also, glycoconjugated bile salts are the abundance components in nature (the ratio of tauroconjugated bile salts to glycoconjugated bile salts is 1:3) in the human intestine. Also steric handicap caused by a sulfur atom of taurine [16]. Is not clear whether bile salt hydrolases recognize the bile acids on cholate group or amino acid groups (glycine/taurine). It may be possible that bile salt hydrolases have evolved to recognize both groups for the enzyme catalysis. The information from available literature suggests that this enzyme recognize their substrates predominantly at the amino acid moieties and not at the cholate moieties [17]. But in a study done by Moser and Savage (2001) [18] revealed that *L. buchneri* JCM1069 expressed hydrolase activity toward taurodeoxycholic acid but not taurocholic acid , taurodeoxycholic acid and taurocholic acid differ at the 7 α-position of their steroid moieties but both have taurine as their amino acid moiety.

Recent investigations have shown that bile salt hydrolyzing Lactobacillus are suggested to manage hypercholesterolaemia through interaction with host bile salt metabolism, Bile salts are synthesized from cholesterol, conjugated with glycine or taurine in the liver, and secreted into the small intestine .The gastrointestinal microbiota have evolved BSH activity to degrade bile salts, playing an essential role in bile detoxification and reducing serum cholesterol levels [19]. Study done by Jiang *et al*., (2010) [20] demonstrated the *Lactobacillus fermentum* capable only of deconjugating taurine- conjugated bile salts, and did not exhibit any BSH activity to glycoconjugated bile salt. As well, *Lactobacillus paracasi* showed activity when grown on MRS agar containing 0.5% (w/v) taurodeoxycholic acid sodium salt [21].

4. CONCLUSION

According to these results, only three isolates exhibited BSH activity when screened in agar plate while ten isolates showed activity in quantitative assay, that means quantitative assay was more accurate and sensitive compared with the direct plate assay for BSH activity determination because from the genetic data, it was indicated that BSH is an intracellular enzyme therefore, its activity was released by cell disruption s either by sonication or other method.

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