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ORIGINAL ARTICLE

BACTERIOCIN-PRODUCING LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL FERMENTED FOOD

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Lactic Acid Bacteria (LAB) isolated from several traditional fermented foods such as "tempeh", "tempoyak" and "tapai" were screened for the production of bacteriocin. One strain isolated from "tempeh" gives an inhibitory activity against several LAB. The strain was later identified as *Lactobacillus plantarum BS2*. Study shows that the inhibitory activity was not caused by hydrogen peroxide, organic acids or bacteriophage. The bacteriocin production was maximum after 10 hours of incubation with an activity of 200 AU/ml. The bacteriocin was found to be sensitive towards trypsin, α-chymotrypsin, β-chymotrypsin, α-amylase and lysozyme.

Key words : *Lactic acid bacteria, bacteriocin,* Lactobacillus plantarum

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Lactic acid bacteria is an important group of industrial microorganism involved in the processing of various fermented food which include vegetables and sausages, dietary adjuncts, probiotics and even cosmetic ingredients (1). It is used as starter culture to improve the texture and the flavour of the products. The ability to inhibit growth of spoilage microorganisms and pathogenic bacteria contribute to the maintenance of hygienic and quality of the products or host health. This inhibitory activity is the result of the metabolic product secreted by these LAB which acts as antimicrobial compounds. These compounds include organic acids, diacetyl, hydrogen peroxide and bacteriocin (2).

Recently, there has been much interest in bacteriocin synthesized by LAB. Bacteriocins, are defined as bioactive peptides or protein with an antimicrobial activity towards gram positive bacteria including closely related species and/or food spoilage and pathogenic bacteria such as *Bacillus cereus*, *Clostridium botulinum*, *Staphylococcus aureus* and *Listeria monocytogenes* (3). The use of bacteriocin or bacteriocin producing culture as potential 'biopreservatives', and possibly for replacing chemical preservatives (4) has received much attention. This is due to current awareness of consumers towards the use of food preservatives. The purpose of this study is to screen lactic acid bacteria isolated from Malaysian traditional fermented food such as "tempeh", "tapai" and "tempoyak" for their ability to produce bacteriocin which exhibit antagonistic activity against indicator strains.

Materials and methods

Strain isolation and screening

The sources of lactic acid bacteria (LAB) were from "tempeh", a fermented soybean cake, "tempoyak", which is a product of fermented durian pulp and "tapai", an alcoholic delicacy made from glutinous rice or cassava.

Lactic acid bacteria strains were isolated from "tempeh", "tempoyak" and "tapai" by weighing 10 grams of each samples and added into 90 ml of bacteriological peptone water (Oxoid). After homogenization, a tenfold serial dilution of the

samples were made and appropriate dilution were streaked on de Man, Rogosa and Sharpe (MRS)-0.14% sorbic acid (MERCK) agar plates. The plates were incubated for 3 days under anaerobic condition at 30°C by placing a gas pack in the anaerobic jar.

Screening of bacteriocin from LAB involves two methods. The first one was to select plates that contain medium density of LAB colonies (30 to 60 colonies) and overlayered them with MRS-soft (0.75%) agar containing indicator strains. The second method was to select the colonies at random, stabbed them onto MRS agar and incubated overnight, anaerobically. The plates were then overlayered with MRS-soft (0.75%) agar seeded with indicator strains. The indicators involved in this study were *Lactobacillus plantarum* 13-2, *Pediococcus acidilactici* 4-46 and *Enterococcus faecalis* N-I-103. These strains were obtained from Microbiology Laboratory, Faculty of Food Science and Biotechnology, UPM. The plates then incubated under anaerobic condition at 30°C. The colonies with antagonistic activity against the indicators were kept in nutrient broth with 15% glycerol and stored in –20°C or streaked on MRS agar for identification and characterization of inhibitory compound.

Identification of lactic acid bacteria

Lactic acid bacteria were characterized by Gram staining and catalase reaction using 30% hydrogen peroxide. The morphology and the motility of the strain were observed under phase contrast microscope. Growth of LAB at 10°C, 15°C and 45°C were done using MRS broth (MERCK). Production of gas from glucose and gluconic acid, arginine hydrolysis, growth in MRS-6.5% broth, growth in acetate agar (5), production of acid and slime from sucrose and final pH in La broth (6) were also determined.

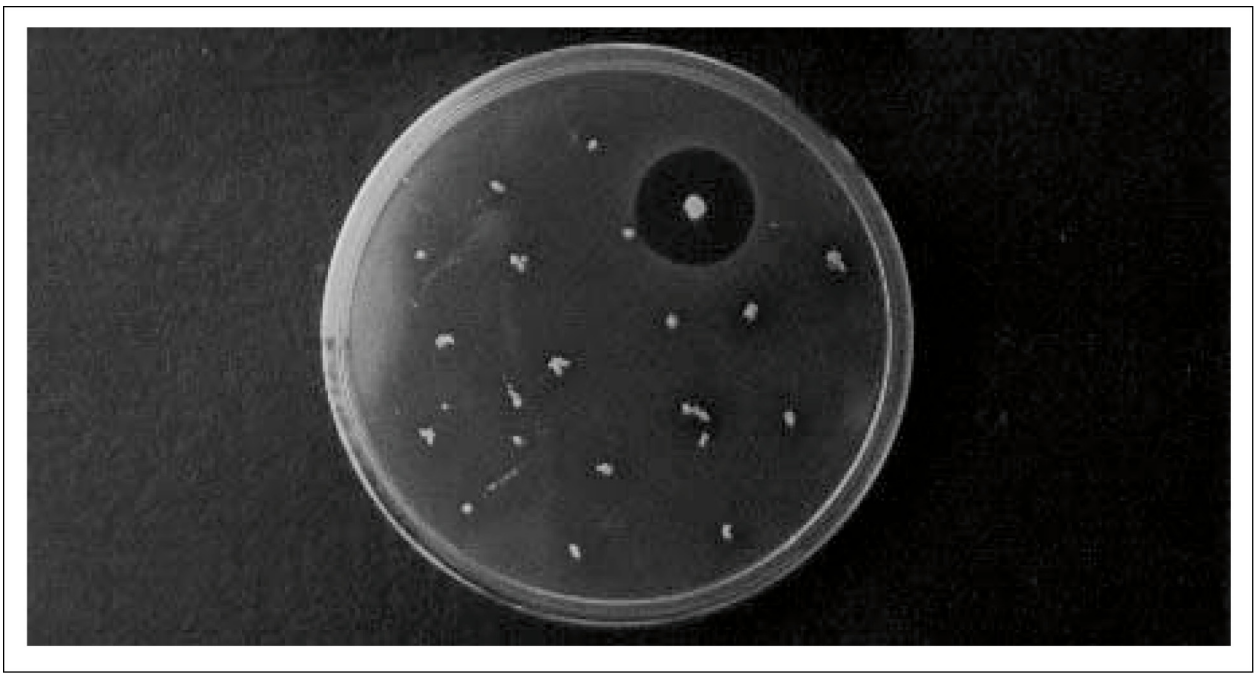
Fermentation of carbohydrates were determined by using API50CHL kit (Biomeriux, France) and the result were analysed by API LAB Plus.

Determining the sources of inhibitory activity

Lactic acid bacteria are able to produce several antimicrobial compounds. These include hydrogen peroxide and organic acids. Bacteriophage may also caused the inhibitory activity. Therefore, it is important to eliminate the inhibition by these compounds in order to ensure that the inhibition was caused by the bacteriocin only. Inhibitory activity by acids can be reduced by using MRS-0.2% glucose (1). Furthermore, preparation of cell free supernatant at pH 6.5 can eliminate the effect of acids produced by lactic acid bacteria against indicator strains.

To eliminate the possibility that hydrogen peroxide may caused the inhibition, a neutralized cell free supernatant were prepared, catalase (5 mg/ml, Sigma) were added into the supernatant before testing for the inhibitory activity. Incubation of

Figure 1: A colony indicated by arrow shows inhibitory zones against indicator strain Enterococcus faecalis *N-I-103.*



producer strain in anaerobic condition may also reduce the effect of hydrogen peroxide against the indicators (7).

The possibility of bacteriophage to cause the inhibitory action was tested by Flip plate method (1). The producer strain was streaked on agar plate and grew anaerobically at 30°C overnight. The agar was then flipped onto the lid of the plates. The indicators were then streaked transversely on the agar and incubated anaerobically at 30°C.

Cell free supernatant

Cell free supernatant were prepared based on methods by Schillinger *et al*. (7). The culture extract of the producer strain were obtained from 18 hours culture grown in MRS broth. The cultures were then centrifuged at 6000 rpm for 10 minutes. The supernatant were adjusted to pH 6.5 using 10N NaOH and filtered through 0.22 μm millipore (Minisart, Sartorius). The resulting supernatant were used for further experiment or stored in –20°C prior to use.

Agar well diffusion assay

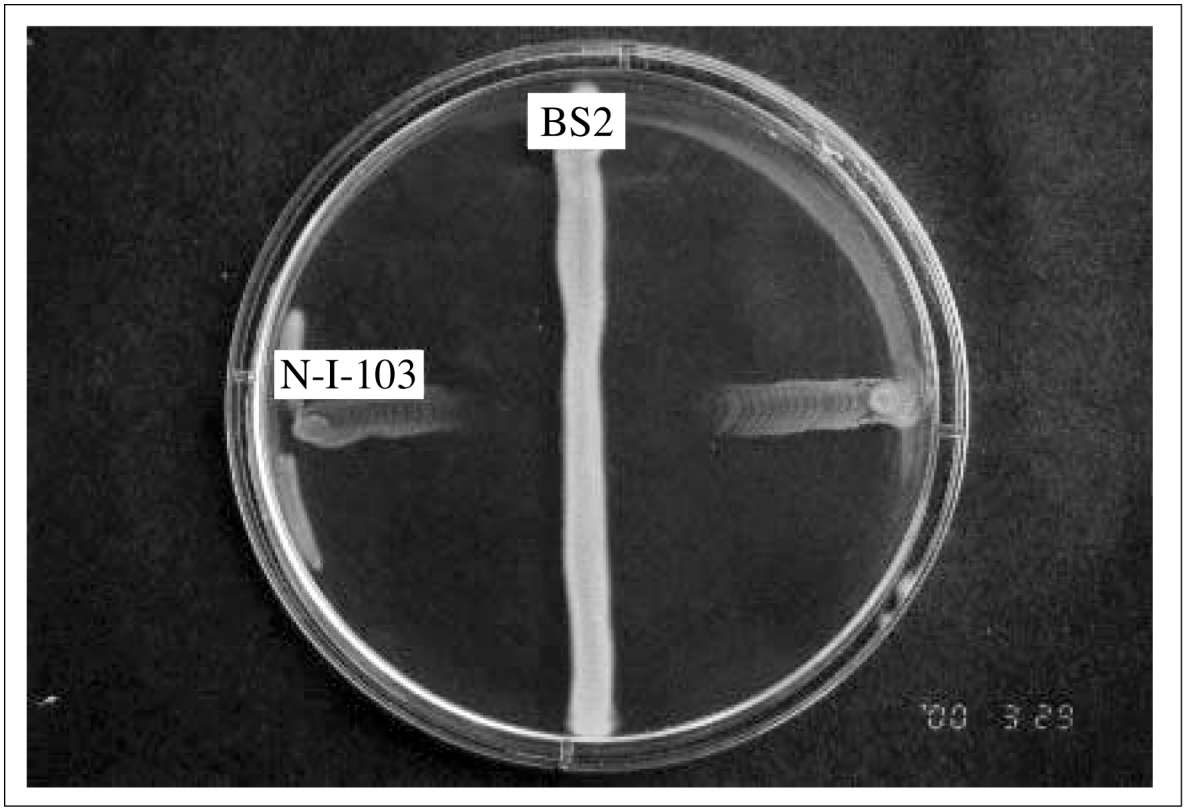
Agar well diffusion methods was suggested by Tagg and Mc Given (8) was used. Wells (5 mm) were made on MRS agar plates using cork borer. 2 to 3 drops of molten MRS agar were added to seal the bottom of the wells and left to hardened. 20 ml of CFS were added into the wells. The plates were kept at –4°C for 2 hours to allow the supernatant to diffuse. The plates were then over layered with MRS-soft (0.75%) agar inoculated with approximately log 7 cfu/ml of indicator (*E. faecalis* N-I-103). The plates were incubated at 37°C overnight.

Activity assay

Serial twofold dilutions of cell free supernatant were prepared using 0.85% sterile saline solution. 20 ml of each of the serial dilution were added into the wells to test for the bacteriocin activity. The activity was assayed by calculating the reciprocal of the highest dilution showing definite inhibition and expressed as Arbitrary Unit /ml (AU/ml) (2).

Time course for bacteriocin production

Figure 2: Flip-plate assay performed to eliminate the involvement of bacteriophage in causing inhibition zone. Therefore, the growth inhibition zone showed by the arrow indicated that the inhibition against Enterococcus faecalis *N-I-103 was due to inhibitory compound produced by* L. plantarum *BS2.*



One percent of overnight culture was inoculated into MRS broth. The culture was then incubated at 30°C. At 2 hours intervals, the growth of the culture were monitored by colony counting at appropriate tenfold dilution to obtain cfu/ml and by optical density at 540 nm. The bacteriocin activity at each time intervals were also determined.

Effect of enzymes towards bacteriocin

The cell free supernatant prepared as above and containing 200 AU/ml of the bacteriocin was used. Supernatant fluid was treated for 1 hour at 37°C with α -amylase, α -chymotrypsin, β-chymotrypsin, lipase, proteinase-K, trypsin, papain and lysozyme (Sigma) at final concentration of 1 mg/ml. The mixture were then heated in boiling water for 3 minutes to denature the enzymes, before testing the bacteriocin activity.

Results and discussion

Isolation and screening

From more than 3000 colonies tested, only one colony was found to produce inhibitory zone. The colony was isolated from "tempeh" and was detected through stab method **(Figure 1)**

Identification of LAB with inhibitory activity

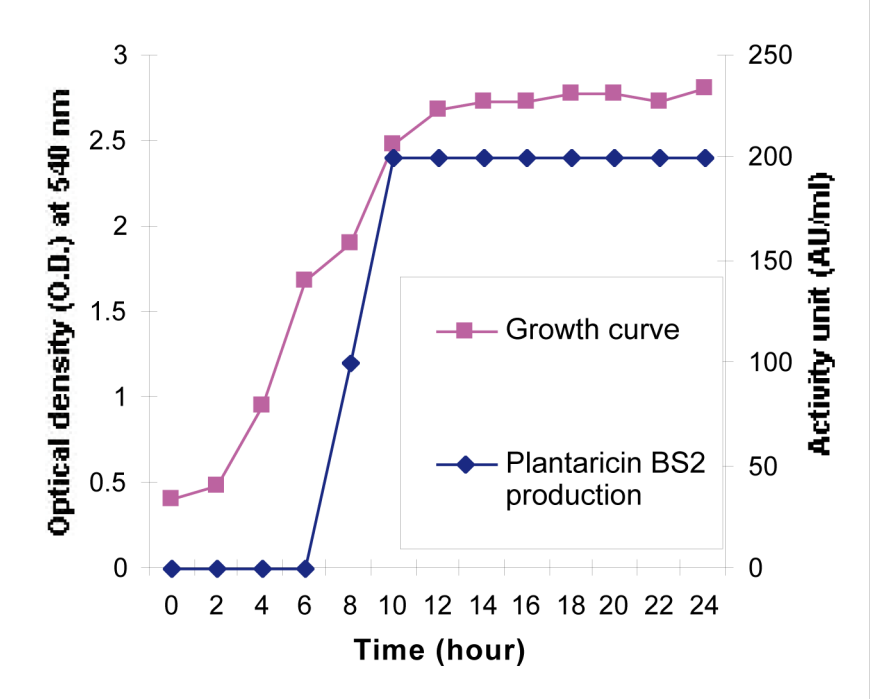
The bacteriocin which exhibited inhibitory activity was found to be Gram positive, catalase negative, short rod and non motile. The culture was able to grow at 10°C, 15°C, 45°C and on acetate agar. It hydrolysed arginine, produced no slime on sucrose agar and gas production from glucose and gluconate. Ability to grow in MRS-6.5% NaCl and in pH 9.6 were other characteristics of this producer strain. These results showed that the isolate was homofermentative *Lactobacillus* species.

The sugar fermentation pattern is presented in **Table 1**. Analysis of the pattern by API LAB Plus shows that it was a *Lactobacillus plantarum* and was given the designation *Lactobacillus plantarum* BS2

Sources of inhibitory activity

Elimination of acid in cell free supernatant does not have any effect on the inhibitory activity of the culture. Addition of catalase reduced the hydrogen peroxide secreted by LAB into H_2O and O_2, therefore reduce the effect of this compound towards the indicators. Positive results after addition of catalase shows that inhibition is not caused by hydrogen peroxide. Clear zone observed from flip plate method **(Figure 2)** shows that bacteriophage is not the source of inhibition since bacteriophage cannot diffuse through agar. Therefore, it can be concluded that the inhibitory action is caused by bacteriocin. This is supported by the effect of proteinase enzymes against the cell free supernatant

Figure 3: Growth curve and bacteriocin production by *L. plantarum* BS2.



(Table 2). Inactivation by all proteinase enzymes tested shows that it is protein or peptide compound. The inhibitory activity of cell free supernatant was also inactivated by addition of α-amylase which showed that this bacteriocin consist of glucidic moieties. The result of enzymes activity against cell free supernatant of *L. plantarum* BS2 are similar to plantaricin UG1 produced by *L. plantarum* UG1 (9)

According to the standard nomenclature for bacteriocin (10) the bacteriocin produced by this bacteria is named as plantaricin BS2. Other reports on plantaricin production by *Lactobacillus plantarum* were by Kato *et al*. (11), Jimenez *et. al*. (12), and Kelly *et al.* (13). However, the comparison of characteristics between plantaricin BS2 and the reported plantaricin are yet to be determined.

Activity assay and time course of bacteriocin production.

The maximum bacteriocin production of 200 AU/ml against *E. faecalis* N-I-103 was achieved after 8 hour of cultivation. Production of plantaricin BS2 started during exponential phase i.e. after 6 hours of incubation. The production reached its maximum when the growth enters the stationary phase **(Figure 3).** This maximum activity was maintained up to 24 hours of incubation.

Application of bacteriocin in food industry

Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* is currently being applied for preservation of dairy products, fish products meat and meat products (14,15,16). Generally, nisin is applied to replace nitrate, a common chemical preservatives used to prevent outgrowth of Clostridia spores as well as other contaminating bacterial pathogens. Currently, bacteriocin had been studied for its suitability to preserve foods. This is due to current concern of the usage of nitrite, which has the potential to form carcinogenic N-nitrosamine. Some biopreservation techniques have now been employed and these involved the introduction of a competitive microflora of lactic acid bacteria (LAB) as protective culturess for chill-stored ready-to-eat meat products, including bacteriocin-producing LAB, and the use of purified anti-listerial bacteriocins added directly as natural food additives. From the results obtained in this study, it is concluded that the *Lactobacillus plantarum* BS2 could serve as a potential source of bacteriocin for use as biopreservative in foods. However, a more detail study on the physical and chemical properties, mode of action and activity against various spoilage and pathogenic bacteria are necessary if the potential of plantaricin BS2 as biopreservative is to be exploited.

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