ISOLATION AND SCREENING OF BIOSURFACTANT-PRODUCING BACILLUS SUBTILIS IN LOCAL TRADITIONAL FERMENTED FOODS

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Abstract- Biosurfactant or microbial surfactants produced by microbes are structurally diverse and contribute in reducing surface and interfacial tension. Apart from being isolated of municipal waste and oil contaminated soil, biosurfactant-producing Bacillus subtilis can also be found in fermented foods as well. In this study, we isolate potential biosurfact-producing Bacillus subtilis from Malaysian traditional fermented foods and do qualitative screening (drop collapse, oil spreading and hemolysis assay) for biosurfactant producer activity.

I. INTRODUCTION

Foods are the basic needs to sustain human life. Various methods have been used to process and preserve foods from ancient time. Fermentation is one of the oldest and widely used food preservation methods in households, small-scale food industries as well as in large-scale industry. Fermented foods generally preserved pleasant flavor, aroma, texture, enhanced nutritive values and good keeping quality under ambient conditions (Law et.al, 2011).

Complex microbiological flora in fermented foods products reflects the broad biological diversity of the natural environments used in the fermentation process, suggesting the potential value of traditional fermented foods as natural resources of bacteria and enzymes for industrial application (Inatsu et.al, 2006).

Bacillus strains have been involved in food fermentation processes. They are able to produce high potential proteases, and their main activity during fermentation is enzymatic proteolysis (Ouoba et. al, 2004). They also have been highlighted as biosurfactant producer for lipopeptide group of surfactin, iturin and fengycin (Mukherjee and Das, 2010).

Biosurfactant had gained interest over chemical surfactant as having low critical micelle concentration and high biodegradibility.

Thus, biosurfactants are well suited for environmental application such as bioremediation and dispersion of oil spills. Biosurfactants also are thought to replace chemically synthetic surfactants in the future, particularly in food, cosmetic and health care industries, as well as in industrial cleaning of products and agricultural chemicals (Mukherjee and Das, 2010).

Previous research had shown that Bacillus subtilis-biosurfactant producer can be isolated from traditional fermented foods across different countries (as in Table 1).

Source	Isolation	Biosurfactant Production	Reference
Doenjang/ Cheonggukjang (Korean fermented soybean paste)	Bacillus subtilis	Surfactin	Lee et. al, 2012
Idli batter (Indian fermented rice)	Bacillus subtilis	Biosurfactant	Joshi et.al, 2008
Soumbala and Bikalga (African fermented locust bean and hibiscus seed)	Bacillus subtilis	Surfactin	Savadogo et.al, 2011
Bikalga (African fermented hibiscus seed)	Bacillus subtilis	Surfactin Bacteriocin	Compaore et.al, 2013
Natto (Japanese fermented soybean)	Bacillus amyloliquefaciens Bacillus subtilis	Iturin A	Murata et.al, 2013

Table 1: Isolation of biosurfactant-producing Bacillus subtilis from various fermented foods source

The objective of this study was to isolate possible Bacillus strain from local fermented foods, which later contribute to biosurfactant production. Then, three types of qualitative screening test was done to identify potential biosurfactant producer.

II. MATERIAL AND METHODS

Isolation of spore-forming bacteria

Seven types of Malaysian traditional fermented foods were collected from Tanah Merah (Kelantan) and Nilai (Negeri Sembilan). 0.1 g of each sample was dissolved in 3.0 ml sterile distilled water, shaken vigorously in waterbath shaker (75°C, 20 mins) to disrupt vegetative microbial cells. The suspension was then plated out on nutrient agar (Oxoid) and incubated at 37 °C for 24h (Chantawannakul et. al., 2002).

A single bacterial colony with different morphological characteristics such as colony elevation, shape, color and surface texture were isolated and transferred onto nutrient agar plates.

Phenotypic identification of isolates

Bacterial single colonies were characterized by their morphological biochemical test (according to Bergey's Manual of Determinative Bacteriology). These included cell shape, Gram staining, presence of spore, starch hydrolysis, Voges-Proskauer Test, citrate test, 6.5% NaCl growth and anaerobic respiration.

The bacterial isolates were then analyzed by analytical profile index, API 50 CHB/E strip (API system, BioMérieux, France). Overnight cultures of isolates were grown on NA plates at 30ºC for 24h. The pure colonies were suspended in API 50 CHB/E medium.

Each 50 tube of the strip was then inoculated with the suspension. Strips were incubated at 30 °C as recommended by manufacturer. The results obtained from color changes of each tube after 24 h and 48 h incubation were recorded before analyzed via API WEB identification software (bioMérieux). Bacillus subtilis ATCC 21332 strain was used as positive control.

Screening for biosurfactant producers

i- Drop collapse test

2.0 µl of mineral oil was added to 96-well micro-titre plate lid. The lid was incubated for 1h at room temperature to ensure a uniform oil coating, and then 5 µl of culture supernatant was added to the surface of oil. After inspecting for 1 minute, biosurfactant-producing cultures gave flat drops were scored as positive and cultures that gave rounded drops were scored as negative, indicating the lack of biosurfactant production (Singh, 2012).

ii- Oil Spreading Assay

30 mL of distilled water was taken in a 25 cm diameter Petri dish to which 1 mL of mineral oil (paraffin/kerosene) was added to the centre, forming a thin membrane. 20 µl of culture supernatant from the broth was then added on top of the oil layer.The Petri dishes were closely observed for a zone of displacement in the oil, and the diameter of displacement was measured (Varadavenkatesan and Murty, 2013).

iii- Hemolysis Assay

Biosurfactant producing capacity was found to be associated with haemolytic activity. (Youssef et al., 2004). Fresh single colony isolates were streaked onto 5% sheep blood agar plates in a sterile environment. The plates were incubated for 48–72 hours at 30°C before visually examined for the presence of clear zones around the streaks (Varadavenkatesan and Murty, 2013).

III. RESULT AND DISCUSSION

Isolation of spore-forming bacteria

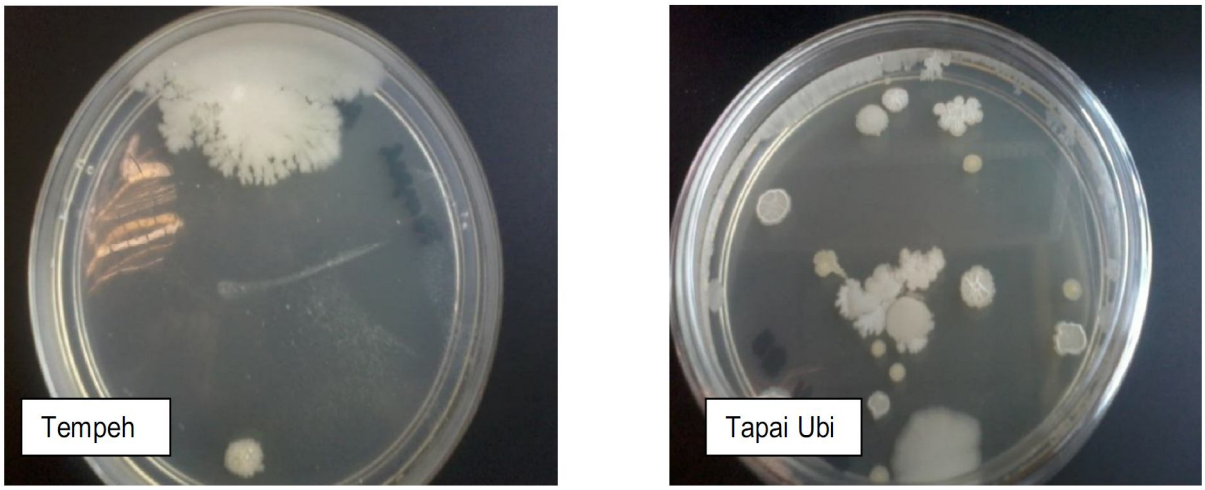


Figure 1: Bacterial multiple colonies from suspension-plating on nutrient agar

Phenotypic identification of isolates

Table 2: Biochemical test of bacterial single colonies
7 types of Malays traditionally fermented foods were collected; tapai ubi, tapai beras, budu kampung (the concentrated ones), pati bilis (budu Cap Ketereh), tempoyak, cincaluk and tempeh. Later, we selected only four bacterial isolates (one isolate from budu kampung, cincaluk, tempeh and tapai ubi) based on their morphological, biochemical and API 50 CHB/E profile. The four isolates were assigned to B. subtilis at profile index greater than 90%, and tentatively were named as BK, CK, TH, and TU for further experiment.

Screening methods for biosurfactant producer
Kiran et al. (2010) recommended that more than one screening method should be included during primary screening to identify potential biosurfactant producers.

Thus, we selected hemolytic activity, oil collapse and oil spreading techniques as the qualitative screening for biosurfactant producers. These methods have strong advantages including simplicity, low cost, quick implementation and use of relatively common equipment that is accessible in a microbiological laboratory (Youssef et. al, 2004; Nasr et. al, 2009; Walter et al., 2010).

i- Drop Collapse Assay
Drop collapse was used as qualitative test to screen for surfactant-producing microbes. This assay is rapid, easy to perform, reproducible and requires little specialized equipment. In presence of surfactant, the culture supernatant drop spreads over hydrophobic surface as the interfacial tension between the droplet and hdydrophobic surface is reduced. In contrast, the droplet remains beaded or rounded in the absence of surfactant, as the polar water molecules are repelled from the hydrophobic surface (Bodour and Miller-Maier, 1998).

ii- Oil Spreading Assay
In the oil spreading technique developed by Morikawa et al. (2000), the area of clearly formed oil displaced circle was measured as the activity of surfactants. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. Hence, this technique is also called as oil displacement activity (Walter et.al, 2010)

The oil spreading method is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample. It can be applied when the activity and quantity of biosurfactant is low (Walter et.al, 2010)

iii- Hemolysis Assay
Biosurfactants can cause lysis of erythrocytes. This principle is used for the hemolysis assay. Positive strains will cause lysis of the blood cells and exhibit a colorless, transparent ring around the colonies (Walter et al., 2010).

According to Carillo et al. (1996), the results were recorded based on type of clear zone observed i.e. α-hemolysis when the colony was surrounded by greenish zone, β-hemolysis when the colony was surrounded by a clear white zone and γ-hemolysis when there was no change in the medium surrounding the colony (Saravanan and Vijayakumar, 2012).

Strain	Drop Collapse	Oil Spreading	Hemolytic Activity
ATCC 21332	Positive	Positive (1.5 cm)	Positive (β-hemolysis)
BK	Positive	Positive (1.8 cm)	Positive (β-hemolysis)
CK	Positive	Positive (1.8 cm)	Positive (β-hemolysis)
TH	Positive	Positive (1.8 cm)	Positive (β-hemolysis)
TU	Positive	Positive (1.5 cm)	Positive (β-hemolysis)

Table 3: Screening of *Bacillus subtilis*-biosurfactant producer



Figure 2: Hemolytic Assay (after 48h)

CONCLUSION

Bacillus subtilis-biosurfactant producer could be isolated from our local fermented foods. Still, the isolates need to undergo further experiments such as molecular identification (16S DNA Sequencing), chemical characterization

(via LC-MS, NMR analysis) and antimicrobial activity to confirm their identities as Bacillus subtilis-biosurfactant producer. Hence, the isolates can exhibit their certain properties which belong to a specific group of biosurfactant.

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