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Preliminary Studies of Halophilic Yeasts Antimicrobial Activities Isolated from Cocoa Bean Pulp towards *E.coli* and *Salmonella spp.*

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***Abstract*—Aims of the research was to determine antimicrobial activities of halophilic yeasts isolated from cocoa bean pulp towards *E. coli* and *Salmonella spp.*. Research done with experimental methods and the data were descriptive-exploratory analyzed. Yeasts isolated from cocoa bean pulp (third days fermentation) and cultured on Malt Extract Agar (MEA). Total yeasts population on Nutrient Agar (NA) with addition of 5 and 10% (w/v) NaCl counted to determine potential halophilic yeast. Antimicrobial activities towards *E.coli* and *Salmonella spp.* determined by diffusion well method on NA. Isolate C2 shown halophilic characteristic with total yeast population of 9.2 x 10^2 cfu/g on 5% NaCl and zero yeasts population on 10% NaCl, while isolate C1 shown better halophilic characteristic with total yeast population of 2.1 x 10^3 cfu/g on 5% NaCl and 1.3 x10^3 cfu/g on 10% NaCl. Antimicrobial activites of selected isolates shown by isolate C1 with 1-2 mm clear zone towards *E.coli* and 2-3 mm clear zone towards *Salmonella spp.* while isolate C2 shown no inhibition towards *E.coli* and 5-10 mm clear zone inhibition towards *Salmonella spp.*.**

Keywords*— Halophilic Yeasts, Cocoa Bean Pulp, Antimicrobial Activities, *E.coli*, *Salmonella spp.

I. INTRODUCTION

Cocoa (*Theobroma cacao* L.) were main ingredient for making chocolate, but it also can used as an important ingredient for cosmetics. The cocoa plant is a perennial tree, and it is originally from the understory of rainforests of South and Central America [1]. Part of the cocoa that can used mainly from the bean after through some certain processes, including fermentation.

The fermentation process of cocoa were very complex microbiological process, because it involves certain microbes such as lactic acid bacteria, spore-forming bacteria, mold and yeasts [2, 3, 4]. The fermentations driven by a complex interplay between raw materials, different microorganisms, and their metabolites.

Pulp is a suitable medium for the growth of microbes, it consists of 82-87% water, 10–15% fermentable sugars (glucose, fructose, and sucrose), 2-3% pentosans, 1–3% citric acid, 1–1.5% pectin, and about 1–2% of other hemicellulosic polysaccharides [5]. In spontaneous fermentation, yeast growth, LAB and acetic acid bacteria derived from contamination of pulp and cocoa beans that play a role in the biochemical changes during fermentation of cocoa [6]. During fermentation, microbial activity in the pulp will produce alcohols, acids and liberate heat. This reaction causes the diffusion of substances into the seed metabolites. This resulted in the formation reaction of flavor, aroma and color [7].

Yeasts are among the most frequently isolated microorganisms from fermenting cocoa beans, especially during the early stages of fermentation. Yeasts are essential in cocoa bean fermentation along with their role in developing the acceptable beans and chocolate flavor [8]. A diversity of yeast species was found, with predominance in the genera now known as *Saccharomyces*, *Hanseniaspora* (anamorph *Kloeckera*), and *Pichia* [1]. Primarily, the yeasts initiate an alcoholic fermentation of pulp sugars to produce mainly ethanol and carbon dioxide, and a vast array of secondary metabolites, such as higher alcohols, organic acids, esters, aldehydes, ketones, sulfur, and nitrogen volatiles, as has been well established for other fermented commodities [9, 10].

Beside producing desired metabolites, cocoa fermentation process will decrease water activity from 0.98-0.99 to 0.72-0.77 after 6 days fermentation [11]. Microorganisms ability to survive in low water acitivity environments were identic with halophilic microorganisms properties [12]. Low water activity also could result by high salinity that shown by others fermented products such pickle [13].

There are high pathogen contamination risks on cacao processing. Contaminations of *E.coli* was highest during drying and storage, with percentages of up to 100% and 89% of positives samples [14]. This is also happen for *Salmonella spp.* contamination risk which has critical period at the end

of fermentation process and the beginning of drying process [11].

At the beginning of fermentation, risk of contamination was lower. Antimicrobial activities at the first stage of cacao fermentation process resulted by yeasts metabolites such as ethanol and organic acids [16]. Presence of phenolics and other phytochemical compound in cocoa fermentation process also give antimicrobial activities [15].

The research objective was to determine the existence of indigenous halophilic yeasts of cocoa beans pulp that has antimicrobial activities towards pathogenic bacteria such as *E. coli* and *Salmonella spp*.

II. Materials and Methods

Cocoa bean (Figure 1) taken from PT PP Bajabang Indonesia, Bandung District. Cocoa bean pulp on third days fermentation diluted on 0.85% NaCl solution and cultured on Malt Extract Agar (MEA) with 100 ppm tetracyclin addition. Halophilic yeasts determined with total yeasts population on Nutrient Agar (NA) with addition of 5 and 10% (w/v) NaCl and incubated at 37ºC for 24h. Antimicrobial activities towards *E.coli* and *Salmonella spp*. determined by well diffusion method on NA [17].

Fig. 1. Cocoa bean (*Theobroma cacao* L.)

III. Results and Discussions

The yeasts isolated on MEA show two different isolates. Table I shown morphology characteristics of isolates C1 was yellow, round and grew on the surface of the media, while isolates C2 was cream, assymetric and grew at the bottom of the media.

TABLE I
Morphology Characteristics of Isolates

Isolate	Colour	Shape	Position
C1	yellow	Round	Surface
C2	cream	Assymetric	Bottom

Two selected yeasts isolate were tested on NA with 5% and 10% NaCl addition. The results on Table 2 showed that only isolates C1 which survived on NA with addition NaCl until 10% with the average population yeasts of 1.3 x 10^3cfu/ml and 2.1 x 10^3cfu/ml yeasts population survived on NA with addition of 5% NaCl. While isolates C2 only survived until 5% NaCl addition with the average yeasts population of 9.2 x 10^2cfu/ml.

TABLE II
Identification of Yeasts Halophilic Characteristics

Isolate	5% NaCl (10^3) (x10^3cfu/g)			10% NaCl (x10^3cfu/g)		
	R1	R2	R3	R1	R2	R3
C1	198	223	211	152	92	134
C2	103	89	84	<30	<30	<30

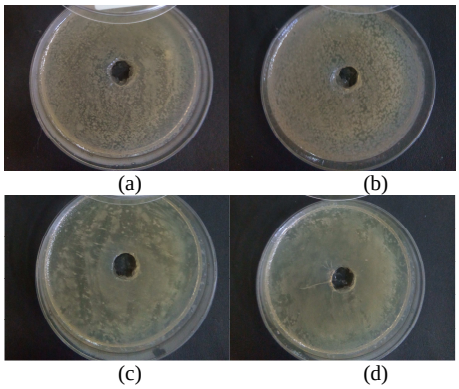
*R = replication

High concentration of saline at the environments could prevent growth of microorganisms. Some of specially adapted organisms found where mostly prokaryotic organisms such as yeasts [17]. *Debaryomyces hansenii* was one of marine yeast that has halophilic characteristics [18]. This halophilic yeasts species also found in food products such as camembert and blue-veined cheese [19].

The mechanism in adapting high salinity of the environment shown by the most extremely halotolerant yeasts species of *Hortaea werneckii* which known as Black yeasts. The black yeasts tolerate high concentration of NaCl by the alternative energy production mechanisms such as glycerophospate shuttle, isovaleryl-CoA dehydrogenase/ electron-transferring-flavoprotein: ubiquinone oxireductase system, or the Na^+ and/orthe H^+ electrochemical potentials and the post-transcriptional regulation [17].

TABLE III
Yeasts Inhibition Abilities Towards Pathogens

Isolate	*E. coli* (mm)			*Salmonella spp*. (mm)		
	R1	R2	R3	R1	R2	R3
C1	1	1	2	2	3	3
C2	-	-	-	10	8	5



(a) (b) (c) (d)

Fig. 2. Antimicrobial activities (a) Isolates C1-*E.coli*, (b) Isolates C2-*E.coli*, (c) Isolates C1- *Salmonella spp*., (d) Isolates C2-*Salmonella spp*.

The results on Table 3 shown the antimicrobial activities of two selected yeasts towards *E.coli* and *Salmonella spp*. (Figure 2). Isolate C2 shown halophilic characteristic with total yeast population of 9.2 x 10^2 cfu/g on 5% NaCl and zero yeasts population on 10% NaCl, while isolate C1 shown better halophilic characteristic with total yeast population of 2.1 x 10^3 cfu/g on 5% NaCl and 1.3 x10^3 cfu/g on 10% NaCl. Antimicrobial activites of selected isolates shown by isolate C1 with 1-2 mm clear zone towards *E.coli* and 2-3 mm clear zone towards *Salmonella spp*. while isolate C2

shown no inhibition towards *E.coli* and 5-10 mm clear zone inhibition towards *Salmonella spp.*.

Antimicrobial activities towards gram negatives bacteria such as *E.coli* and *Salmonella spp.* could caused by the production of organic acid and hydrogen proxide [20]. Yeasts known could produce organic acids (hexanoate, octanoate, and decanoate) and protein that could inhibit the growth of pathogenic bacteria [21]. Beside that, there were also available the antimicrobial proteins that could act as an antimicrobial agent [22]. Moreover, yeasts also have ability in producing sulphur dioxide that could inhibit the growth of bacteria [23].

Halophilic yeasts with antimicrobial activities has wide range prospect in food production. Utilization in salted fermented products such as cheese or pickle, useful to produce desired flavor and also reduce pathogenic bacteria contamination risks.

IV. Conclusions

Isolate C1 shown better halophilic characteristic with total yeast population of 2.1 x 10^3 cfu/g on 5% NaCl and 1.3 x10^3 cfu/g on 10% NaCl. Antimicrobial activites of isolate C1 was 1-2 mm clear zone towards *E.coli* and 2-3 mm clear zone towards *Salmonella spp.*, while isolate C2 shown no inhibition towards *E.coli* and 5-10 mm clear zone inhibition towards *Salmonella spp.*.

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