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Microbial Consortium Synergism for Promising Freshwater Culture Probiotic

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Abstract. Freshwater culture productivity can be improved by intensification. One of the most effective intensifications is the addition of probiotic agents. *Bacillus subtilis*, *Lactobacillus lactis*, and *Saccharomyces cerevisiae* have great potential as probiotic by improving water quality in freshwater culture. In the microbial consortium, the member of microbes has to have the ability to form synergism relationship to perform probiotic functions. Synergism interactions can be based on material transfer that relates to the energetic, cell to cell communication or physical protection. Advantage of microbial consortium synergism as a probiotic candidate has higher effectiveness and causes a broad spectrum effect than a single culture. The purpose of this study was to establish a synergism relationship between *B. subtilis*, *L. lactis*, and *S. cerevisiae*as probiotic candidates in freshwater culture by testing using cross streak method and to know growth profile between themicrobes. The results showed that synergism occurred between all microbes depicting none inhibition zones between isolates. A half logarithmic phase at the growth curve can be known so microbial consortium probiotic formed.

INTRODUCTION

Currently, the freshwater culture industry is growing with the increasing demand for fish, since 60% of protein needs are from fish meat. Freshwater culture productivity can be improved through extensification and intensification. Intensification method is more popular because the extensification method is limited by the land area factor. The addition of antibiotics is one of the intensification methods that is often used by fishermen, but antibiotics can cause serious problems, such as resistance of pathogen and dangerous to the food chain in the environment [1][2][3]. So, the most effective intensification is the addition of probiotic agents [1].

Probiotics are a single or mixed culture of microbes added in a particular amount that benefits fish cultivation [5]. Some advantages of probiotic use is that it is safer, not pathogenic to fish, no accumulation in the food chain, the reproduction mechanism can decrease repeated usage and no resistance to target organisms [6]. Microbial culture of probiotic can be from bacteria, yeast, or harmless fungi [1]. Probiotic can be obtained quickly by combining several potential probiotic microbes through a consortium. Microbial consortium probiotics are more effective and have a broad spectrum than only a single culture probiotic [7].

Probiotic candidate, such as *Bacillus subtilis*, *Lactobacillus lactis*, and *Saccharomyces cerevisiae*, can improve water quality in freshwater culture media. *B. subtilis* and *S. cerevisiae* can reduce ammonia compounds through oxidation and assimilation mechanism [8][9][10]. According to [11], *Lactobacillus* can reduce nitrogen compounds, such as ammonia, nitrites, and nitrates. Also if probiotic candidates could be formed a consortium then the water quality will be improved and also improve fish health through changes of bacteria composition in water and sediment [12].

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The main requirement for the microbial consortium probiotic is synergism relationship between microbes, where microbes have the same ability, complement each other and work together in a group [13][14]. In the microbial consortium, interaction can occur through cell-to-cell communication or signals from a compound that can bind to proteins on the cell surface [15]. This study was to determine synergism interaction between *B. subtilis*, *L. lactis* and *S. cerevisiae* by cross streak method and to determine growth profile between microbes, so the expected results can be used as a reference for starter culture of fresh water culture probiotic.

MATERIALS AND METHODS

Materials

Isolates used in this study were *Bacillus subtilis*, *Lactobacillus lactis* and *Saccharomyces cerevisiae* from Microbiology and Biotechnology Laboratory, Department of Biology, Faculty of Science, Institut Teknologi Sepuluh Nopember (ITS) Surabaya. Materials used were Nutrient Agar (NA), Trypticase Soy Agar (TSA), de Man Rogosa and Sharpe (MRS agar), Yeast Malt Agar Agar (YME agar), yeast extract, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, and molasses. The instruments used were Laminar Air Flow (LAF), Rotary Shaker and UV-Vis Spectrophotometer in the Microbiology and Biotechnology Laboratory, Department of Biology, Faculty of Science, Institut Teknologi Sepuluh Nopember (ITS) Surabaya.

The Subculture *Bacillus subtilis*, *Lactobacillus lactis*, and *Saccharomyces cerevisiae* on Nutrient Agar (NA)Medium

Each microbe was subcultured in different enrichment media with inoculating loop. A loopful of *B. subtilis* were inoculated on Trypticase Soy Agar (TSA). A loopful of *L. lactis* were inoculated on de Man Rogosa and Sharpe agar (MRS agar). A loopful of *S. cerevisiae* were inoculated on Yeast Malt Extract Agar (YME agar). Isolates were incubated at room temperature for 24 to 48 hours [16][17][18]. Viable isolates of each microbe were subcultured in Nutrient Agar (NA) medium by streak plate method. These cultures were incubated for 24 hours (bacteria) and for 2 to 3 days (yeast) at room temperature. Viable isolates were used as stock cultures in Nutrient Agar (NA) medium [17].

Synergism Test between Isolate

Synergism test by cross streak method on Nutrient Agar (NA) medium was carried out by a scheme shown in Fig 1. One isolate was chosen to be straight vertically streaked in Nutrient Agar (NA), then other isolates cross streaked on both sides. The inoculum was incubated for 2 to3 days at room temperature. The result was shown by the presence or absence of inhibitory zones formed on cross streak point. The synergism relationship between isolates was indicated by no inhibition zone, while antagonism relationship was indicated by inhibition zone [19][20].

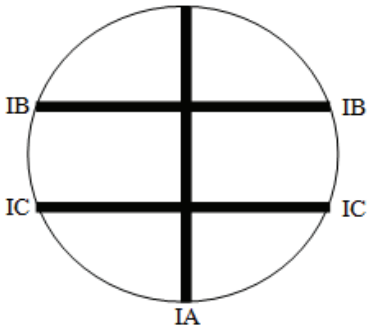


FIGURE 1. Synergism test with cross streak method. Isolate A (IA), Isolate B(IB), Isolate(IC) [20]

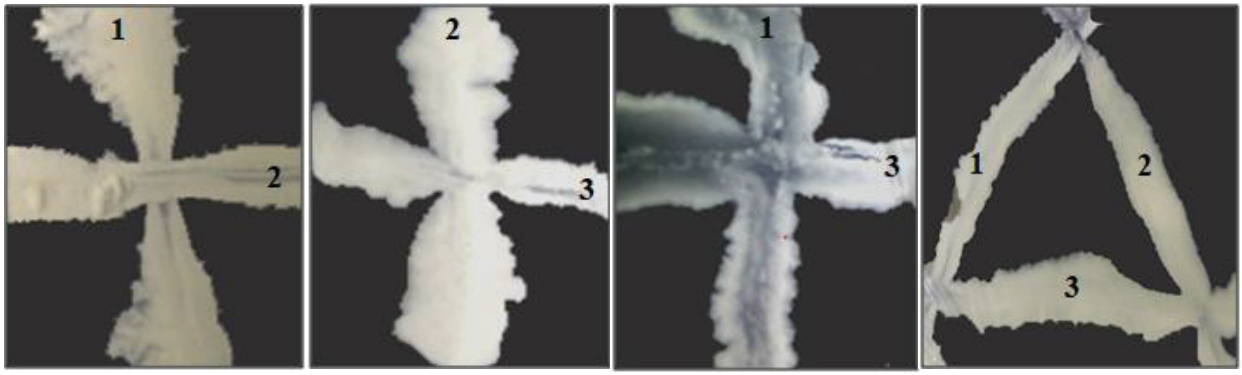
Growth Curve between Isolates

Growth profile of microbe consortium was determined using a spectrophotometer. The growth medium composition for the culture consisted of yeast extract 0.2%, KH_2PO_4 0.2%, $(NH_4)_2SO_4$ 1%, $MgSO_4 \cdot 7H_2O$ 0.1% and molasses 1%. A loopful of each microbe was inoculated in each 20 mL sterile growth medium. The inoculum was incubated on 130 rpm rotary shaker at room temperature for 24 hours. After 24 hours of incubation, the culture liquid's optical density was measured using UV-Vis spectrophotometer at λ = 600 nm. Each of viable inoculum 10% was inoculated to 180 ml growth medium. The inoculum was incubated on 130 rpm rotary shaker at room temperature. Cell density was measured using UV-Vis spectrophotometer at λ = 600 nm every 2 hours for 48 hours [13][16][21].

RESULTS AND DISCUSSION

Synergism Test

Synergism test was carried out to determine the relationship between isolates because if antagonism interactions occur by depicted inhibition zone, the isolate cannot form consortium [20]. Synergism test results are shown in Fig. 2.



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

FIGURE 2. Synergism test by cross streak method (a) between *B.subtilis* (1) and *L.lactis* (2), (b) between *L.lactis* (2) and *S.cerevisiae* (3), (c) between *B.subtilis* (1) and *S.cerevisiae* (3), (d) between *B.subtilis* (1), *L.lactis* (2) and *S.cerevisiae* (3).

The advantage of cross streak method is that it is easier and faster to obtain results because all metabolism compounds will be produced during the test period [22][23].

TABLE 1. Result of synergism test

Isolate	*B.subtilis*	*L.lactis*	*S.cerevisiae*
B.subtilis	X	+	+
L.lactis	+	x	+
S.cerevisiae	+	+	x

Notes:
Sign (+) represents a synergistic isolate, sign (-) is an antagonistic isolateandsign (x) is not a synergism test.

Based on Fig. 2a and Table 1, it can be seen that *B.subtilis* and *L. lactis* had a synergism relationship, indicated by no inhibition zone formed between the isolates. *B.subtilis* is a positive catalase while *L.lactis* is not a positive catalase. *L.lactis* inhibited growth and activity of other microbes by synthesizing acid and H_2O_2 .*B.subtilis* can produce catalase enzyme that can hydrolyze H_2O_2 into oxygen and water, so two microbes can be synergistic in the same environment [24]. Besides that, the antimicrobial peptide, like subtilosin (bacteriocin) secreted by *B. subtilis,* is not antagonistic to the *Lactobacillus* group, this statement agrees with the result from [25], that subtilosin can increase the growth and viability of *L. reuteri* and *L.acidophilus*.

L. lactis and *S. cerevisiae* had a synergism relationship, indicated by no inhibition zone formed between the isolates (Fig. 2b and Table 1). *S.cerevisiae* can only use simple carbon sources, such as glucose, fructose and galactose. So that *S.cerevisiae* can get glucose and galactose as lactose hydrolysis product by *L.lactis*[26]. *S.cerevisiae* grows better in an acidic condition provided by *L.lactis*. Also, *S.cerevisiae* can produce metabolite compounds, such as organic compound and CO_2, that can decrease the level of H_2O_2 compounds. CO_2 can balance oxygen in the environment, while organic compounds can hydrolyze H_2O_2 into non-toxic compounds with peroxidase. Growth of *L. lactis* can be increased with vitamins (riboflavin / Vit B12) synthesizing by S.cerevisiae [27][28].

Based on Fig. 2c and Table 1, it can be seen that *B.subtilis* and *S.cerevisiae*had a synergism relationship, which is indicated by no inhibition zone formed between the isolates. *B.subtilis* can produce several enzymes, such as amylase, glucosidase, and galactanase, that can degrade complex carbohydrates (in the form of starch) into a simple sugar source so that it can be used as an energy source by *S. cerevisiae. S. cerevisiae* is unable to synthesize enzymes for degrading complex carbon sources[29][30].

The absence of inhibitory zones was formed from synergism test by the three isolates, which showed that the three isolates had synergism relationship (Fig. 2d and Table 1). *B.subtilis* and *L. lactis* can produce starch hydrolysis enzyme, that provides carbon source (glucose) for *S.cerevisiae*. It can improve bacteria performance to produce enzymes through glucose consumption because the highest glucose concentration in the environment can inhibit enzyme synthesis by the bacterium [31].

Growth Profile between the Isolates

Growth profile of the microbe consortium is used as a reference for microbial consortium production [32]. The growth profile of the consortium results is shown in Fig.3.

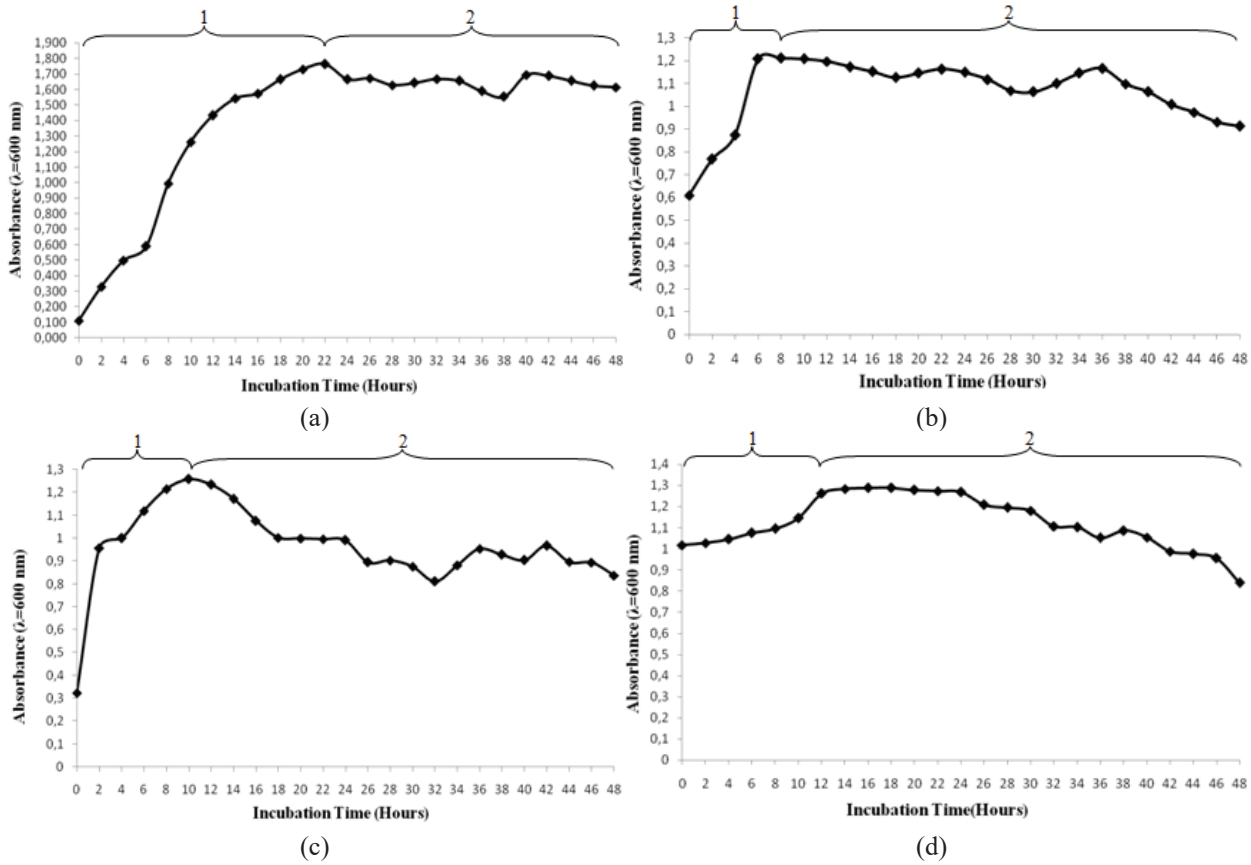


FIGURE 3. Growth profile (a) between *B.subtilis* and *L.lactis*, (b) between *L.lactis* and *S.cerevisiae*, (c) between *B.subtilis* and *S.cerevisiae*, (d) between *B.subtilis*, *L.lactis,* and *S.cerevisiae*. (1) Log phase, (2) Stationary phase.

Each growth curve of consortium in Fig. 3 shows no lag phase but directly shows the log phase. This condition occurred because the medium used had the same composition as the starter culture medium for each isolate. According to [33], if the inoculum is grown on a growth media the same as in the acclimatization process, the lag phase can be eliminated. Based on Fig. 3, it is shown the half-log phase difference in each consortium, which is sequenced in the half-log phase from Fig. 3a to Fig. 3d occurred at the 12th hour (OD_{600}= 1.435), 8th hour (OD_{600}= 0.874), 6th hour (OD_{600}= 1.119) and 6th hour (OD_{600}= 1.076). The half log phase based on Fig. 3b to Fig. 3d is obtained faster than Fig. 3a because one of the components of the consortium is a yeast that is classified as eukaryotes. *S. cerevisiae* has a larger size than the two bacteria so that the turbidity level in the medium is faster and the half log phase occurs quickly [34]. Probiotic production needs a half-log phase in the growth curve because its cell division continues to the highest biomass and cell in a healthy condition[32]. Rapid cell growth in half-log phase is influenced by the growth media, such as pH, temperature, humidity and nutrition [33].

CONCLUSION

B. subtilis, *L. lactis* and *S. cerevisiae* had synergism interactions that were proven by no inhibition zone between the isolates. Thus the three microbes can be used as a probiotic microbial consortium in freshwater culture. The growth profiles between the isolates were made as references for starter culture of the probiotic microbial consortium. Recommendation of this study is to add other synergism test method to obtain a higher level of accuracy and further research of the microbial consortium is expected to be carried out to determine the effects of probiotics on freshwater culture.

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