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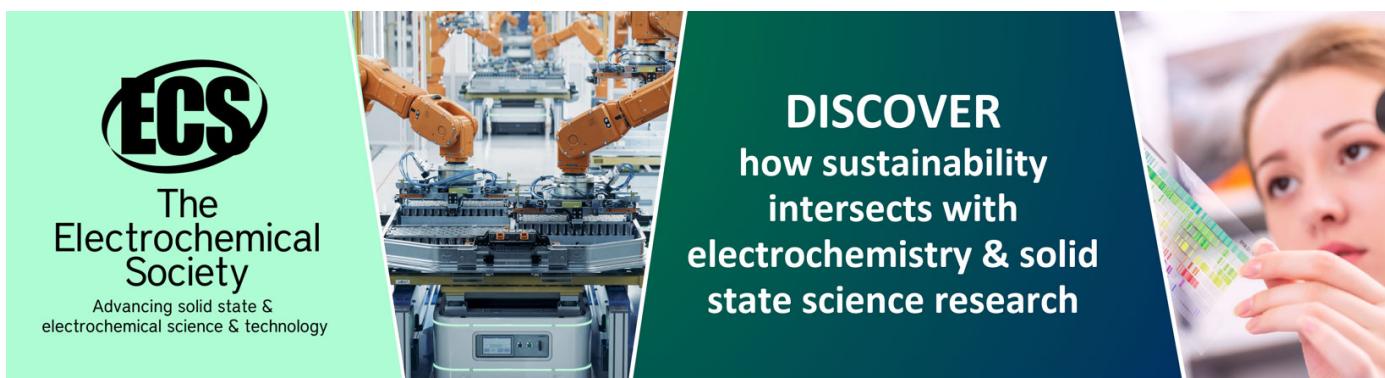
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Screening of Marine Microalgae Collected from Wakatobi as Anti-Tyrosinase

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Abstract. Three microalgae isolates from Wakatobi waters collected by LIPI have been cultivated, ie LIPI 13-2-AL018, LIPI 13-3-AL066, and LIPI 13-2-AL072. Furthermore, the isolates were grown to create a growth curve, this is to determine the harvest time of biomass. Each isolate had different biomass harvest times: LIPI 13-2-AL018 and LIPI 13-2-AL072 isolates were harvested on the 14th day, while LIPI 13-2-AL066 on day 12. After harvesting, the biomass was dried, destroyed the cell wall with sonicator. Extraction of secondary metabolite compounds was performed using three types of solvents, namely: ethanol, ethanol + n-hexane (1: 1), and diethyl ether. Based on the results of inhibition testing of tyrosinase it was found that the biggest inhibition was derived from extract of LIPI-13-2-AL066 isolate, using ethanol-hexane solvent.

1. Introduction

Tyrosinase is an enzyme that catalyzes the oxidation of phenolic substrates into o-quinons, which are then polymerized into brown, red or black pigments [1]. Melanin synthesis takes place inside these melanosomes through the conversion of tyrosin by the enzyme tyrosinase, first into dihydroxyphenylalanine (DOPA) and then into DOPA-quinone, and subsequently into eumelanin, which bestows black-brown color, and pheomelanin, which bestows yellow-red [2]. Already many synthetic chemical compounds are used in cosmetics to inhibit the tyrosinase enzyme. In addition, natural tyrosinase inhibitors are generally considered to be free of harmful side effects and can be produced at reasonable low cost [3]. One of the potential biological resources for tyrosinase inhibitors is microalgae. The Indonesian science institute (LIPI), had explored microalgae from the Wakatobi islands in Southeast Sulawesi. At present these microalgae isolates become a collection of LIPI.



2. Materials and Methods

2.1. Cultivation and Identification of microalgae

2.1.1. Culture medium

The media used are two types, namely IMK and AF6. Making the modified IMK media begins with weighing 0.252 g of IMK (Daigo, Japan), then added filtered 1 L of sea water. The media is sterilized for 15 minutes, temperature 121 ° C before use. In the sterile media, 0.015 mL of vitamin B12, 0.015 mL of thiaminHCl, 0.015 mL of biotin and 0.002 mL of Vitamin B6 were added.

Making AF6 media was carried out by weighing 0.14 g NaNO₃, 0.004 g NH₄NO₃, MgSO₄·7H₂O 0.03 g, KH₂PO₄ 0.01 g, K₂HPO₄ 0.005 g, CaCl₂·2H₂O 0.01 g, CaCO₃ 0.01 g, Fe Citrate 0.002 g, Citric Acid 0.002 g, enter 1 L distilled water, 1 mL PIV metal, the homogeneous medium and pH adjusted to 6.6, then sterilized by autoclaving. Furthermore, the sterile solution was mixed with 0.01mL of Vitamin B6, 0.01 mL of Thiamin HCl, 0.01 mL of Vitamin B12, 0.02 mL of Biotin.

2.1.2. Cultivation and Growth Curve

Microalgae stock is prepared on 50 mL medium. Subsequently transferred in a 250 mL in volume. The 250 mL microalgae is a stock that will be cultivated. Microalgae cultivation uses a 500 mL culture bottle, aerated at room temperature and given 24 hours of lighting. The microalgae cell growth rate was observed every day until it reached the stationary phase using a Shimadzu UV-Vis spectrophotometer at a wavelength of 680 nm. Harvesting is done when the microalgae population reaches a maximum or approaches maximum density. The growth curve is made by plotting time (days) with its absorbance value.

2.2. Extraction of microalgae bioactive

Wet biomass is destroyed by the cell wall using sonicators [4,5]. Then the extract was centrifuged at a speed of 6000 rpm for 10 minutes to take the supernatant. The wet extract is evaporated with a Rotary Vacuum Evaporator so that a thick extract is obtained. The extraction process of bioactive compounds uses three types of solvents, namely: ethanol, ethanol + n-hexane (1: 1), and diethyl ether.

2.3. Preliminary Test of Tyrosinase Enzyme

2.3.1. Optimization of L-DOPA substrate concentration

A 50 mM phosphate buffer solution of pH 6.8 as much as 600 µL was pipetted into four test tubes, then added 200 µL of L-DOPA substrate solution with various concentrations of 1, 2, 2.5, and 3 mM respectively. The solution is incubated at 25°C for 10 minutes. The enzymatic reaction starts with adding 200µL of tyrosinase solution. The formation of dopachrom was measured using a UV-Vis spectrophotometer at 475 nm.

2.3.2. Standard Curve of Kojic Acid Inhibition to Tyrosinase

One of the functions of kojic acid is as a tyrosinase inhibitor which is quite popular in the cosmetic industry as a skin whitening agent. The result of inhibition by kojic acid in various concentrations of tyrosinase is a comparison of inhibition by microalgae extract. Kojic acid concentrations used were: 10 (µg / mL), 20 (µg / mL), 30 (µg / mL), 40 (µg / mL), 50 (µg / mL) and 60 (µg / mL).

2.4. Measurement of anti-tyrosinase activity

Tyrosinase-inhibition activity of the extract microalgae was performed by using L-DOPA as a substrate [6]. Analysis of inhibition performance of microalgae extracts was seen by changes in color intensity through UV-Vis spectrophotometer measurements. The measured absorbance is the absorbance of dopakrom product formation, the result of oxidation between L-DOPA by the enzyme tyrosinase, the beginning of the formation process of melanin.

Tyrosinase inhibition activity was calculated by comparing the absorbance of the sample (50mM phosphate buffer solution, 2.5 mM L-DOPA, extract and tyrosinase solution) with positive control

solution (50 mM phosphate buffer solution, 2.5 mM L-DOPA, tyrosinase solution, and kojic acid). The value of tyrosinase inhibition activity was used to compare the effect of microalgae extract concentration with the percentage concentration of kojic acid used to inhibit the enzyme tyrosinase

3. Results and discussion.

3.1. Cultivation and characteristics of microalgae

Microalgae cultivation uses 3 isolates, namely: LIPI 13-2-AL018, LIPI 13-2-AL066, and LIPI 13-2-AL072, which come from the collection of Bioenergy and Bioprocess Laboratories, LIPI Biotechnology Research Center in Cibinong. The media used for all three isolates were: LIPI isolates 13-2-AL018 using AF6, while LIPI isolates 13-2-AL066, LIPI 13-2-AL072 used IMK media. The six isolates were observed using a Leica microscope, to ensure that the isolate was a single isolate. Based on the results of the observation, the images of each microalgae isolate were as follows:

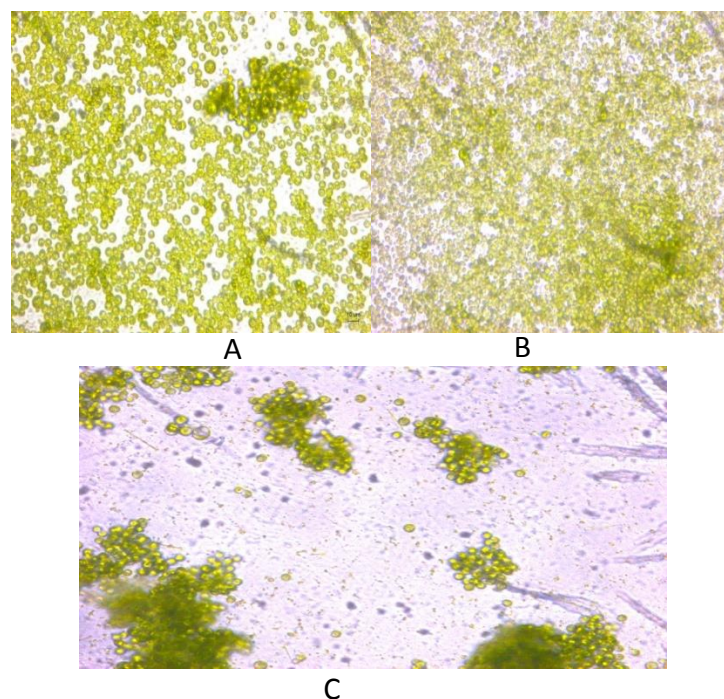


Figure 1. Microalgae morphology: (A) LIPI 13-2-AL018; (B) LIPI 13-2-AL066; (C) LIPI 13-2-AL072

Observation of microalgae cell morphology LIPI 13-2-AL018 is round in shape forming a chain, yellowish green in color and has a larger diameter than other isolates. LIPI Isolate 13-2-AL072 has almost the same shape as LIPI 13-2-AL018 isolate but its diameter is smaller, tends to grow and does not colonize. Whereas LIPI 13-2-AL066 isolates are round, green and tend to grow in colonies.

3.2. Growth dynamics of microalgae

Microalgae cultivation is carried out in bottles connected with aeration hoses and 24-hour lighting. Microalgae growth was observed every day using UV-Vis Shimadzu spectrophotometer [7]. Measurements were carried out 2 repetitions for each isolate, starting from day 0 to the stationary phase

Isolate of LIPI 13-2-AL018 and LIPI 13-2-AL072 get the most cell density on day 14 so that culture was harvested on day 14. While for LIPI isolates 13-2-AL066 the most cell density occurred on the day to 12, so that was harvested on the 12th day (Figure 2).

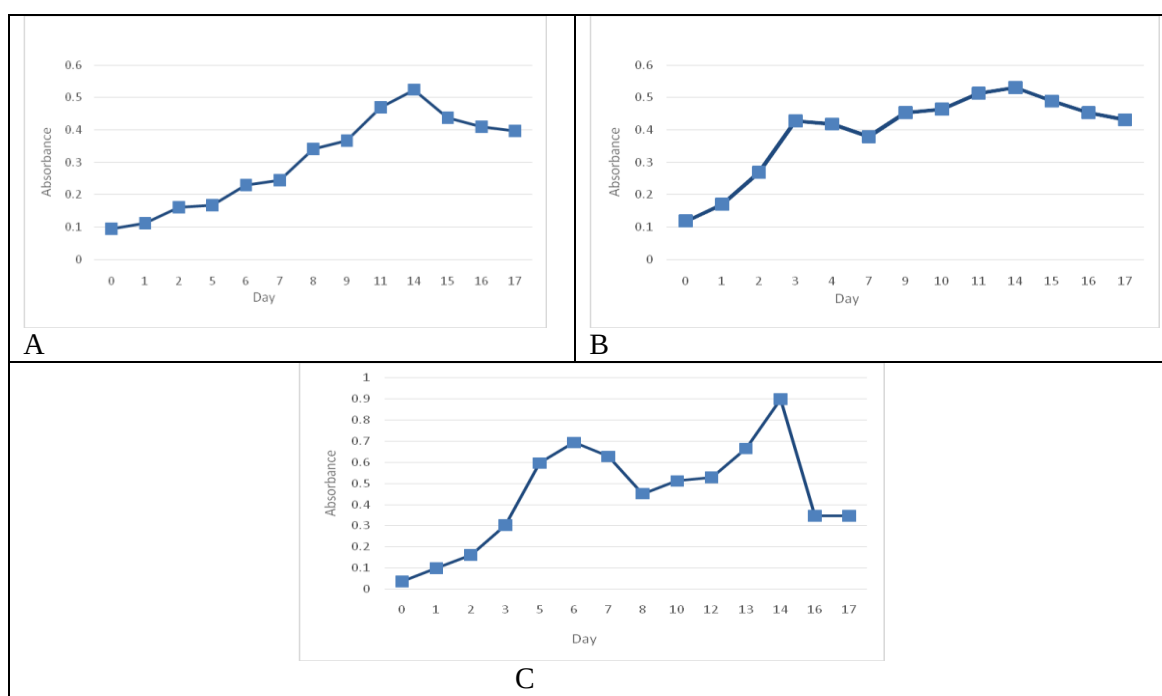


Figure 2. Microalga growth stage: (A) LIPI 13-2-018; (B) LIPI 13-2-066; and (C) LIPI 13-2-072

After knowing the microalgae growth stage, the cultivation process was carried out again by taking stock from a volume of 250 mL. Cultivation was made 2 times each with a volume of 500 mL. Microalgae dry biomass for each isolate obtained as follows:

Tabel 1. Microalgae dry biomass weight

ISOLATE	DRY BIOMASS (gr/mL)
LIPI 13-2-AL018	0.12155 $\pm$ 0.04
LIPI 13-2-AL066	0.0374 $\pm$ 0.02
LIPI 13-2-AL072	0.1772 $\pm$ 0.05

3.3. Inhibition of Tyrosinase activity

3.3.1. Determination of the Optimum Concentration of L-DOPA Substrate

Determination of the optimum concentration of L-DOPA substrate was carried out by varying the concentration of L-DOPA, namely: 1 mM, 2 mM, 2.5 mM, and 3 mM. Based on the results obtained that the optimum concentration of L-DOPA is 2.5 mM (figure 3).

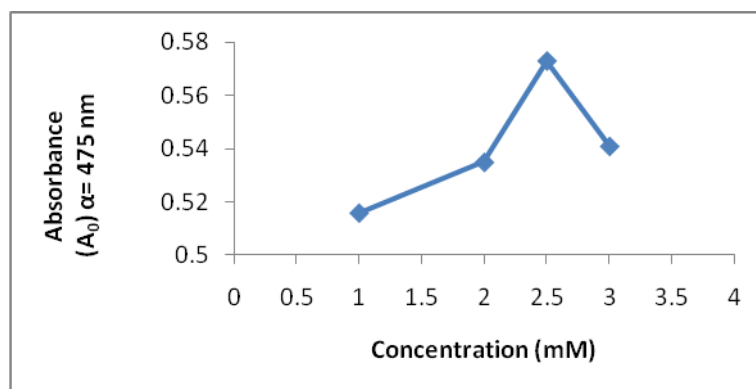


Figure 3. The optimum concentration of L-DOPA

3.3.2. Inhibition of Tyrosinase activity by Kojic Acid

Tyrosinase inhibition test of kojic acid was carried out by varying the concentration of kojic acid, ie 10 $\mu\text{g} / \text{mL}$, 20 $\mu\text{g} / \text{mL}$, 30 $\mu\text{g} / \text{mL}$, 40 $\mu\text{g} / \text{mL}$, 50 $\mu\text{g} / \text{mL}$ and 60 $\mu\text{g} / \text{mL}$. Based on the observation of absorbance values at various concentrations of kojic acid obtained the equation $y = -0.0052x + 0.6725$ and $R^2 = 0.9648$ (figure 4)

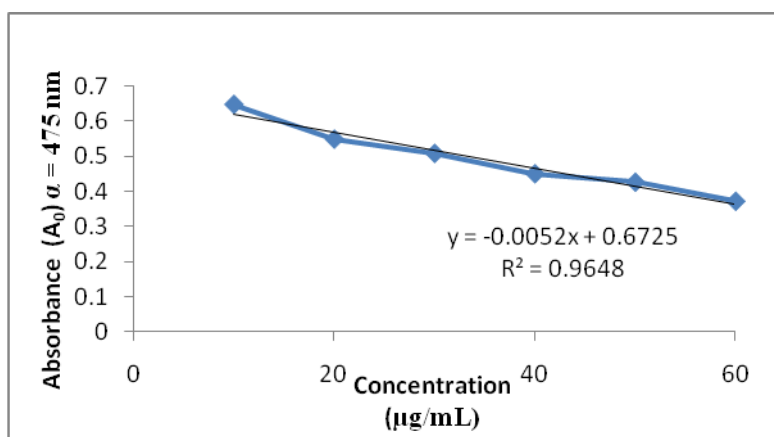


Figure 4. Standart curve of inhibition of tyrosinase activity by kojic acid

3.3.3 Inhibition of Tyrosinase Activity by microalgae extract

Extraction of microalgae bioactive compounds was carried out with three types of solvents, namely: ethanol, ethanol + n-hexane (1:1) and diethyl ether. Furthermore, each crude extract was tested for inhibitory activity against the tyrosinase enzyme (table 2).

Table 2. Inhibition of tyrosinase activity by microalgae extract

Isolate	Volume	inhibition is equivalent to the concentration of kojic acid		
		ethanol+n-hexane	Ethanol	Diethyl ether
LIPI 13-2-018	50 μL	6.2 ± 0.98	19.1 ± 3.95	14.8 ± 0.14
LIPI 13-2-066		34.1 ± 5.93	18 ± 1.83	2.3 ± 0.84
LIPI 13-2-072		9.3 ± 1.41	19.2 ± 1.27	9.2 ± 1.83
LIPI 13-2-018	100 μL	21.6 ± 0.42	22.8 ± 4.94	20 ± 2.68
LIPI 13-2-066		43.8 ± 11.73	23.7 ± 4.52	4.7 ± 1.41
LIPI 13-2-072		15.6 ± 3.81	21.5 ± 2.26	11.6 ± 0.7

The tabel above are obtained by substituting the absorbance value of the sample (crude extract) into the standard curve equation of kojic acid, $y = -0.0052x + 0.6725$. The biggest inhibition value was obtained in LIPI-13-2-066 isolate with ethanol + n-hexane (1: 1) solvent variation, which is equivalent to 43.8 $\mu\text{g} / \text{mL}$ kojic acid.

According to Burnett et al., 2010 the level of kojic acid that is safe to use in cosmetic products up to a concentration of 1%. On the other hand, it was explained that at 100 mmol/L (14.2 mg/mL), kojic acid had a 76.7% + 1.1% inhibitory effect on mushroom tyrosinase [8]. Bioactive compounds from microalgae need to be purified so that the equivalent is less than 43.8 $\mu\text{g} / \text{mL}$ of kojic acid.

4. Conclusions

Based on the results of inhibition testing of tyrosinase was found that the biggest inhibition value was obtained in LIPI-13-2-066 isolate with ethanol + n-hexane (1: 1) solvent variation, which is equivalent to 43.8 $\mu\text{g} / \text{mL}$ kojic acid

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