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Optimization media from low-cost nutrient sources for growing *Spirulina platensis* and carotenoid production

Optimasi media dengan sumber nutrisi murah untuk pertumbuhan dan produksi karotenoid Spirulina platensis

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Ringkasan

Spirulina platensis *adalah sianobakteria yang menghasilkan berbagai senyawa bioaktif bernilai ekonomi tinggi antara lain karotenoida. Untuk memproduksi karotenoida dari biomassa sel* S. platensis *secara efisien, perlu ditetapkan komposisi media mineral dan bahan organik kompleks yang optimal dari sumber nutrisi yang murah.* Spirulina platensis *yang ditumbuhkan dalam media serum lateks dari pabrik lateks pekat dengan suplemen garam-garam mineral tertentu diharapkan produktif dalam menghasilkan karotenoida. Tujuan penelitian adalah menetapkan komposisi media yang optimal untuk pertumbuhan dan produksi karotenoid serta mengidentifikasi jenis senyawa karotenoid dalam biomassa sel* S. platensis*. Sianobakteria ini ditumbuhkan dalam media kompleks mengandung serum lateks pekat (5%, v/v) dengan suplemen nutrisi berupa makronutrien dan mikronutrien selama 10 minggu di dalam ruangan dengan aerasi dan penyinaran lampu TL 20 W pada jarak 50 cm. Komposisi makronutrien diformulasi untuk memberikan sebelas macam variasi nisbah C:N:P:Mg. Sebagai pembanding digunakan media sintetik Aiba & Ogawa. Hasil penelitian menunjukkan bahwa pertumbuhan* S. platensis *mencapai puncak setelah diinkubasikan selama delapan minggu. Dari 11 komposisi media mengandung lateks yang diuji, pertumbuhan* S. platensis *terbaik adalah yang ditumbuhkan dalam media formula dengan nisbah C:N:P:Mg= 1:3:0.3:0.2 menghasilkan 0,350 g biomassa/L, sedikit lebih rendah dibandingkan dengan menggunakan media sintetik Aiba & Ogawa yang menghasilkan 0,407 g biomassa/L selama 8 minggu. Walaupun kandungan biomassanya lebih rendah, media formula tersebut menghasilkan karotenoid lebih tinggi dibandingkan dengan media sintetik Aiba & Ogawa. Kandungan karotenoid tertinggi pada biomassa yaitu sebesar 2.866 mg/kg diperoleh pada media dengan nisbah C:N:P:Mg=1:2:0.3:0. Analisis ekstrak biomassa dengan TLC menunjukkan adanya dua-enam jenis karotenoida, salah satunya adalah β - karotena.*

Summary

Spirulina platensis is a cyanobacteria producing several bioactive compounds such as carotenoids which are economically valuable. To produce carotenoids in *S. platensis* biomass efficiently, it is necessary to define an optimum medium composition consisting of mineral salt and organic complex derived from low-cost nutrient sources. *Spirulina platensis* grown on complex media containing latex serum from concentrated latex factory, supplemented with salt minerals might produce high yielding carotenoids. The objective of this research is to define media composition for optimum growth and carotenoid production of *S. platensis* and to identify carotenoid compounds from biomass of the algae. *S. platensis* was grown on media containing latex serum from latex concentrate factory (5%, v/v), macroelements and microelements, for 10 weeks at a room aerated and illuminated by 20 W TL lamp at 50 cm distance. Microelements were formulated at a certain amount to give eleven combinations of C: N: P: Mg. The Aiba & Ogawa synthetic medium was used as a reference medium. The optimum growth of *S. platensis* had reached after eight-week incubation. Among eleven media composition containing latex serum examined, best growth on a formulated medium with a ratio of C: N: P: Mg = 1:3:0.3:0.2 yielding 0.350 g biomass/L This amount was slightly lesser than those on synthetic Aiba & Ogawa medium that yields 0.407 g biomass/L, after eight-week incubation. Although the biomass production was lower than that of synthetic

Aiba & Ogawa medium, the formulated media gave higher carotenoid content. The highest carotenoid content in biomass was 2.866 mg/kg biomass obtained from a medium with ratio of C: N: P: Mg = 1: 2: 0.3: 0. Thin layer chromatography (TLC) analysis of biomass extract showed the presence of two-six carotenoid compounds, in which one of them is β- carotene.

[*Key words* : *Spirulina platensis*, latex serum, waste water, carotenoids, β-carotene]

Introduction

Natural rubber is one of the most important commodities in Indonesia since it could generate foreign exchange revenue of $US 1.101 million per year, contributing employment opportunities for 12 millions of people, and providing important raw materials for local industries (Direktorat Jenderal Perkebunan, 2000). In 2001, the total rubber production reached 1.5 million ton and about 5% was in the form of latex concentrate. Although latex concentrate contributes relatively small amount against total rubber production, the effluent generated from concentrated latex factory could pollute environment compared to solid rubber, as indicated by COD, BOD, and N total that could reach 25,000, 10,000 and 4,000 ppm, respectively (Darussamin *et al.,* 1988). The latex serum was obtained during centrifugation process of ammoniated field latex. The latex serum is rich in proteins, lipids, carbohydrates, and minerals such as Mg, P, K, Ca (Jacob *et al.*, 1993). The utilization of latex serum as components of microbiological media could reduce the volume as well as increase the added value of the effluent.

Spirulina platensis is a cyanobacteria or a blue-green microalga that is relatively fast growing, having large size (2 x 110 μm), capable of growing in high salinity and alkalinity with the presence of carbonate bicarbonate and inorganic nitrogen (Aiba & Ogawa, 1977). The biomass of *S. platensis* has been recognized to be a "wonderful food health" since it contains high protein (Umesh & Sheshagiri, 1984) and various bioactive compounds such as essential fatty acids (linoleic and γ-linolenic acids) (Borowitzka, 1988b; Cohen *et. al*., 1987), essential amino acids, B-complex vitamins (riboflavin, cyanocobalamin, thiamin, nicotinic acid), biopigments (phycocyanin and chlorophyll a) (Achmadi *et al.*, 2002), and carotenoids (Cohen, 1997). The β-Carotene content in biomass is important for food health, pharmaceuticals, and cosmetics industries and is thus potential to be produced in large scale (Borowitzka, 1988a).

In previous experiments, latex serum had been used for growing media of micro-fungi (*Absidia* sp. and *Rhizopus* sp.) and *S. platensis* to produce single cell protein (SCP) and γ-linolenic acid (GLA) (Tri-Panji *et al.*, 1994; Tri-Panji *et al*., 1995). Latex serum supplemented with salt minerals could enhance growth of *S. plantensis* (Tri-Panji *et. al*. 1996). Protein hydrolysates contained in latex serum is considered promotive of *S. platensis* growth. Singh *et al.* (1995) found that protein hydrolysates could increase the biomass of *S. platensis* and GLA deposited in its biomass**.** Medium composition is one of the major contributor to the productivity of microbial metabolites in their biomass such as GLA (Botha *et al*., 1997) and protein (Eijifor *et al*., 1995). The use of low-cost nutrition such as latex serum that is economically unutilized and even to be waste, together with technical grade chemicals in optimal medium was considered capable of reducing production cost of *S. platensis* biomass. Tanticharoen *et al.* (1993) reported that the use of low-cost nutrients such as secondary treated starch wastewater reduced production cost in *Spirulina* culture and improved the effluent quality.

To obtain high concentration of bioactive compounds in *S. platensis* biomass, the media should support growth for both biomass yield and particular bioactive compounds that will be recovered. However, only few studies have been conducted to examine the influence of composition of culture media on the production of carotenoids from *S. platensis.*

The present investigation is to determine the optimum composition of mineral medium containing latex serum for production of biomass as well as carotenoids and to identify the carotenoid compounds present in *S. platensis* biomass.

Materials and Methods

Preparation of inoculum

The cyanobateria *S. platensis*, used in this experiments is a culture collection of the Biotechnology Research Unit for Estate Crops, Bogor. The cyanobacteria was maintained in an aerated culture of Aiba & Ogawa synthetic liquid medium, at room temperature (28-32 ^{o}C) under continuous lighting with 20 W tungsten light (TL) lamp at 50 cm distance. Subculturing was done every three months. Inoculum was prepared from 2-month-old culture growing on the same media until optical density (OD) of 1.0 at λ 480 nm. Ten percent (v/v) culture suspension of *S. platensis* was used in each inoculation to the media investigated.

Growth condition

Spirulina platensis was grown on a medium containing macro and micro nutrients with a combination of composition given in Table 1 and latex serum (5%, v/v) was added at 28-32^{o} C to a 2.5 L Erlenmeyer flask containing 1 L medium each, and illuminated from upper side with 20 W TL lamp at 50 cm distance. The cultures were aerated for 10 weeks. The manipulation of media were made by adjusting their nutrient composition as described in Tables 1, 2, supplemented with Triple Super Phosphate (TSP), potassium nitrate, and magnesium

Table 1. Composition of modified Aiba & Ogawa medium of *S. platensis* containing salt mineral and latex serum

Tabel 1. Komposisi media S. platensis *mengandung garam mineral dan serum lateks modifikasi dari Aiba & Ogawa*

Na_2CO_3 *	12.5	g/L
NaCl*	1.75	g/L
$FeSO_4$ *	0.005	g/L
Latex serum**	50	mL/L
Micro nutrient from stock solution	2	mL/L
Composition of micro nutrient in stock solution	100	mL
H_3BO_3	0.286	g
$MnSO_4$	0.150	g
$ZnSO_4$	0.020	g
Na_2MoO_4	0.003	g
$CuSO_4$	0.007	g

Note (*keterangan*) : *) Technical grade (*Angka teknik*)
**) Collected from Cikumpay latex-concentrate factory, West Java, Indonesia (*Lateks konsentrat, koleksi pabrik Cikumpay, Jawa Barat, Indonesia*)

Table 2. Modification of media at various weight ratios of C: N: P: Mg
Tabel 2. Modifikasi media dengan beberapa rasio berat dari C:N:P:Mg

Medium (*Media*)	Weight ratio of C: N: P: Mg (*Rasio berat dari C:N:P:Mg*)
I	1: 2 : 0 : 0
II	1: 2 : 0 : 0.2
III	1: 2 : 0.3: 0
IV	1: 2 : 0.3: 0.2
V	1: 2 : 1.2: 0.2
VI	1: 2 : 0.3: 0.7
VII	1: 2 : 1.2: 0.7
VIII	1: 1 : 0.3: 0.2
IX	1: 1.5: 0.3: 0.2
X	1: 2.5: 0.3: 0.2
XI	1: 3 : 0.3: 0.2
XII ∗synthetic medium of Aiba & Ogawa (*media sintetik Aiba & Ogawa*)	12: 1.7: 0.4: 0.2

Note (*keterangan*)*:* ∗synthetic medium (*media sintetik*)

sulfate (technical grade) at a certain concentration to give a final C:N:P:Mg ratio as mentioned in Table 2. These nutrient ratios represent an adjustment of C at fixed amount with various combination of N, P, and Mg both at low, moderate and high concentration. Synthetic medium of Aiba & Ogawa (1977) in axenic culture of *S. platensis* was used as a reference medium. The compositions such as macronutrient (g/L): $NaHCO_3$ 13.6, Na_2CO_3 4, $MgSO_4.7H_2O$ 0.2, $CaCl_2$ 0.03, $FeSO_4.7H_2O$ 0.01, K_2HPO_4 0.5, KNO_3 2.5, $K_2SO_4$1, NaCl 1, EDTA 0.08. Micronutrient (g/L): H_3BO_3 2.86, $MnSO_4.H_2O$ 1.55, $ZnSO_4.7H_2O$ 0.22, Na_2MoO_4 $2H_2O$ 0.03, $CuSO_4.5H_2O$ and $CoCl.6H_2O$ 0.01. The growth was monitored every week by measuring OD of culture filtrate at 480 nm. For carotenoid analysis, the biomass was harvested after eight weeks by filtration with two layers of muslin cloth and sun dried for three days, followed by oven dried at 60^{o}C for three hours, weighted, and pulverized with a warring blender.

Carotenoids analysis

Carotenoids compounds were isolated according to AOAC (1990) with modification of the extraction solvent using solution mixture of hexane/acetone/ethanol (1:1:1 v/v), followed by removing lipids with saponification. Powdered biomass (0.1-0.5 g) was added to 15 mL extraction solvent in a test tube. The test tube was capped with aluminum foil and shaken vigorously with a vortex for 5-10 min, and incubated over night in the dark. The extracted carotenoids were removed into Erlenmeyer flask, and heated in the oven at 60˚C to vaporize the extraction solvent. Saponification was carried out by adding 1 mL 40% KOH in methanol to the residue and stored in the dark for one hour. The mixture was poured into a 50 mL volumetric flask and adjusted up to level with 15 mL hexane and 10% sodium sulfate, shaken vigorously and stored in the dark for one hour. Top layer of the mixture was

decanted for carotenoid analysis by reading the absorbance at λ 444 nm. For standard solutions, β-carotene standard (Sigma) was dissolved in hexane, adjusted to serial concentration (10-1000 ppm) and its absorbance was read at the same wavelength. Carotenoids of the sample were calculated as β-carotene by plotting against the curve of standard solutions.

Identification of the carotenoid compound was carried out using thin layer chromatography (TLC). Sample (50 µL) was bloted at a silica gel plate (5x10 cm) and eluted by a mixture of hexane/ acetone (60:40 v/v).

Results and Discussion

Growth and carotenoid production

The growth curve of *S. platensis* on media with eleven combinations of phosphate, magnesium, and potassium nitrate and in a reference medium of Aiba & Ogawa are shown in Figure 1. In general, an increase in growth (expressed as OD) occurred during incubation period for 8 weeks. After an optimum growth was achieved during eight-week incubation, the extension of incubation period tends to decrease the growth. Different concentrations of P and Mg (Media I-VI) affected the growth of *S. platensis* (Figure 1a). Medium V (medium with high P and low Mg), and medium VI (medium with low P and high Mg) could not support the growth of *S. platensis*. A sharp decrease in biomass was apparent during incubation period (Figure 1a). This may be due to the cell lyses since detrimental effect of the high level supplementation of P or Mg on growth of *S. platensis*. Thus, the biomass from the two media could not be harvested for carotenoid analysis due to the very low yield of biomass recovery. Improvement of growth was obtained when P and Mg were given in approximately proportional amount and moderate level (medium IV). Different concentration of N and K in the form of potassium nitrate affected the growth of *S. platensis* (Figure 1b). Growth of *S. platensis* increases markedly with the increasing supplementation of potassium nitrate in the growth media with P and Mg at moderate and proportional level (media VII, IX, X, XI, and XII).

Production of biomass and carotenoids was investigated from ten media compositions containing latex serum, nine of them were capable of producing carotenoid in *S. platensis* biomass (Table 3). Besides Aiba & Ogawa synthetic medium, the highest biomass yield was obtained in medium XI yielding 0.350 g/L. The biomass production of *S. platensis* in medium XI was slightly inferior to Aiba & Ogawa synthetic medium (medium XII). This medium contains the highest level supplementation of potassium nitrate (2.4 g/L). In general, potassium nitrate for microorganisms is a readily available source of nitrogen that could increase growth performance and carotenoid deposition.

Carotenoid content on the basis of dry weight biomass varies with media used (Table 1). It was shown that without P and Mg supplementation to the medium (medium I), the carotenoids was synthesized in very low concentration (207.58 mg/g dry weight biomass). Supplementation of media with Mg (medium II) and P (medium III) could significantly increase their carotenoid production, such as 804.17 and

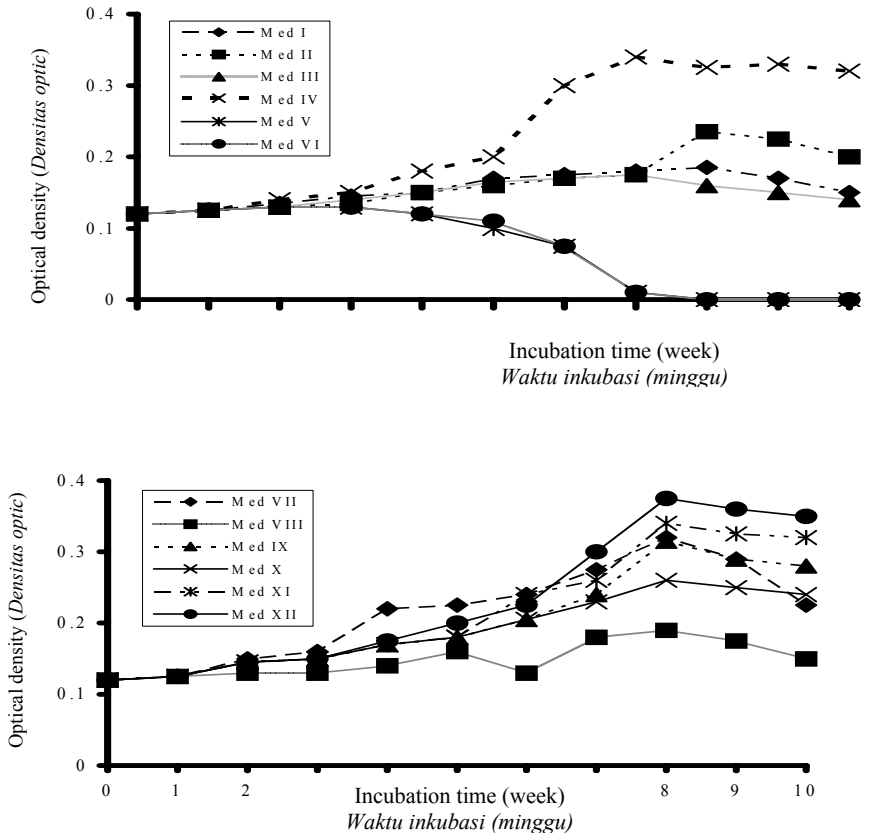

Figure 1. Growth curve of *S. platensis* on 11 compositions of medium containing latex and on synthetic medium (XII) as a reference at λ = 480. C:N:P:Mg ratio in: medium I 1: 2 : 0: 0; medium II 1: 2 : 0 : 0.2; medium III 1: 2 : 0.3: 0; medium IV 1: 2 : 0.3: 0.2; medium V 1: 2 : 1.2: 0.2; medium VI 1: 2 : 0.3: 0.7; medium VII 1: 2 : 1.2: 0.7; medium VIII 1: 1 : 0.3: 0.2; medium IX 1: 1.5: 0.3: 0.2; medium X 1: 2.5: 0.3: 0.2; medium XI 1: 3 : 0.3: 0.2; synthetic medium of Aiba & Ogawa 12: 1.7: 0.4: 0.2.

Gambar 1. *Kurva pertumbuhan* S. platensis *pada 11 komposisi medium yang mengandung lateks dan medium sintetis (XII) sebagai pembanding pada λ = 480 C:N:P:Mg dalam : medium I 1: 2 : 0: 0; medium II 1: 2 : 0 : 0.2; medium III 1: 2 : 0.3: 0; medium IV 1: 2 : 0.3: 0.2; medium V 1: 2 : 1.2: 0.2; medium VI 1: 2 : 0.3: 0.7; medium VII 1: 2 : 1.2: 0.7; medium VIII 1: 1: 0.3: 0.2; medium IX 1: 1.5: 0.3: 0.2; medium X 1: 2.5: 0.3: 0.2; medium XI 1: 3 : 0.3: 0.2; medium sintetis dari Aiba & Ogawa 12: 1.7: 0.4: 0.2.*

Table 3. Carotenoids content and biomass yield of *S. platensis* grown on various media composition

Tabel 3. Kandungan karotenoid dan hasil biomassa dari pertumbuhan S. platensis yang tumbuh pada berbagai komposisi media

Medium *Media*	Biomass concentration (*Konsentrasi biomassa*) (g/L medium)	Carotenoid content of biomass (*Kandungan karotenoid dalam biomassa*) (mg/kg dry basis, *berat kering*)	Carotenoid production (*Produksi karotenoid*) (mg/L medium)
I	0.101	207.58	0.021
II	0.062	804.17	0.050
III	0.063	2866.44	0.181
IV	0.128	1184.16	0.152
VII	0.077	1338.52	0.104
VIII	0.057	n.d	n.d
IX	0.092	1159.36	0.107
X	0.104	719.06	0.075
XI	0.350	1150.50	0.402
XII ∗synthetic medium of Aiba & Ogawa (*medium sintetik dari Aiba & Ogawa*)	0.407	937.26	0.381

Note (*keterangan*) : n.d, not detected (*tidak terdeteksi*)

2866.44 mg/g dry biomass respectively, but a slightly decrease in their biomass. Magnesium supplementation together with P (media IV and VII) increased both biomass and carotenoid production. The combination P and Mg such as in medium IV supplemented with an increasing level of potassium nitrate (media IX, X, and XI) increased the biomass production, yielding significant amount of carotenoids. Thus, optimal supplementation of C, N, P, Mg, for both production of biomass and carotenoid was in the ratio of 1: 3: 0.3: 0.2 (w/w), i.e. medium XI. This medium seems to have a good nutrient balance and thus support the optimal growth and carotenoid production of *S. platensis.*

Microbial carotenoids are produced as secondary metabolites. According to Brock & Madigan (1995) the formation of secondary metabolites is extremely dependent on the composition of the media, and the metabolite formation does not always occur in parallel to growth or biomass production. Botha *et al*. (1997) also recorded that media supporting good biomass production of mucoralean fungi was not concomitant with their ability to produce GLA. The highest carotenoid content was obtained in medium III (2.866 mg/kg biomass dry weight). This result is lower than that reported by Cohen (1997) which reaches 6.480 mg/kg biomass dry weight.

Spirulina platensis grown on medium XI produced less carotenoids if it is expressed in dry biomass basis compared to that on medium III. However, total carotenoid that could be potentially recovered per litre medium and per production cycle was higher at medium XI (0.4 mg carotenoids/L medium). This was due to the high biomass production in medium XI (0.350 mg biomass/L medium). Although the productivity of biomass was lower than that of synthetic medium (Aiba &Ogawa synthetic medium), the productivity of carotenoid seemed higher in media III, IV, VII, IX, and XI. Achmadi & Tri-Panji (2000) also found that synthetic medium yielded biomass density higher than

that on latex serum medium, but the recovery of biopigment phycocyanin was less. In order to commercialise this product using the media as mentioned, an effective large-scale culture system should be carried out. Borowitzka (1994) reported that tubular photobioreactor was to be one of the most potential large-scale micro-algal culture system.

Identification of carotenoid compounds

Thin Layer Chromatography (TLC), using hexane/acetone (60: 40 v/v) as an eluent had been carried out for initial identification of carotenoid compounds. Figure 2 shows the TLC chromatogram of the carotenoid compounds of *S. platensis* biomass harvested from media I, II, III, IV, VII, VIII, IX, XI & XII at different incubation time. Samples were prepared from hexane extract after treated by saponification with potassium hydroxide. Other substance such as gliserides, phospholipids, fatty acid (as their derivatives), and chlorophylls were separated in water-soluble fraction and remaining carotenoid compounds were soluble in the organic solvent.

The TLC chromatogram showed a clear yellow spot at the Rf of 0.96-0.98 corresponding to authentic β-carotene (Figure 2, node f). This result showed that all *S. platensis* biomass from different culture time investigated could produce β-carotene. Borowitzka (1988b) reported that carotenoids of microalga were rich of β-carotene and astaxanthin. The remaining spots (Figure 2, node a, b, c, d, & e) having different Rf value (Table 4) may be carotenoid compounds as well, but were not identified in this study. Biomass harvested from different media composition during 21-33 days produces 4-5 spots of carotenoid compounds. Except in medium VIII, the other formulated media gave similar results (Table 4). Spot a, b, c, d, and e have a low Rf value compared to β-carotene. It means that the other carotenoid compounds (spot a, b, c, d, and e) consisting molecules with more polarity than β-carotene. Cohen (1997) and Harborne (1987) showed that the carotenoid compounds such as γ-carotene, lutein, zeaxantine, violaxatine and cryptoxantine having lower Rf value compared to that of β-carotene on TLC analysis using non polar eluant.

One of the difficulties in the identification of other carotenoid compounds in this study was the lack of Rf value reference at the same TLC conditions. However, the TLC analysis could provide valuable information for the separation of various carotenoid compounds using column chromatography for commercial production. β-Carotene with the Rf value 0.96-0.98 would possibly be released firstly from column chromatography when the same developing solvent and stationary phase were used. Then, β-carotene was recovered by solvent evaporation. TLC analysis could be used to confirm the level of purity of the recovered product. During the extraction of the carotenoids, valuable by-product resulted from saponification treatment i.e. fatty acids could be produced in the form of its salt. The fatty acids could be released from its salts by adding mineral acid and the free fatty acids could be recovered by extraction with organic solvent.

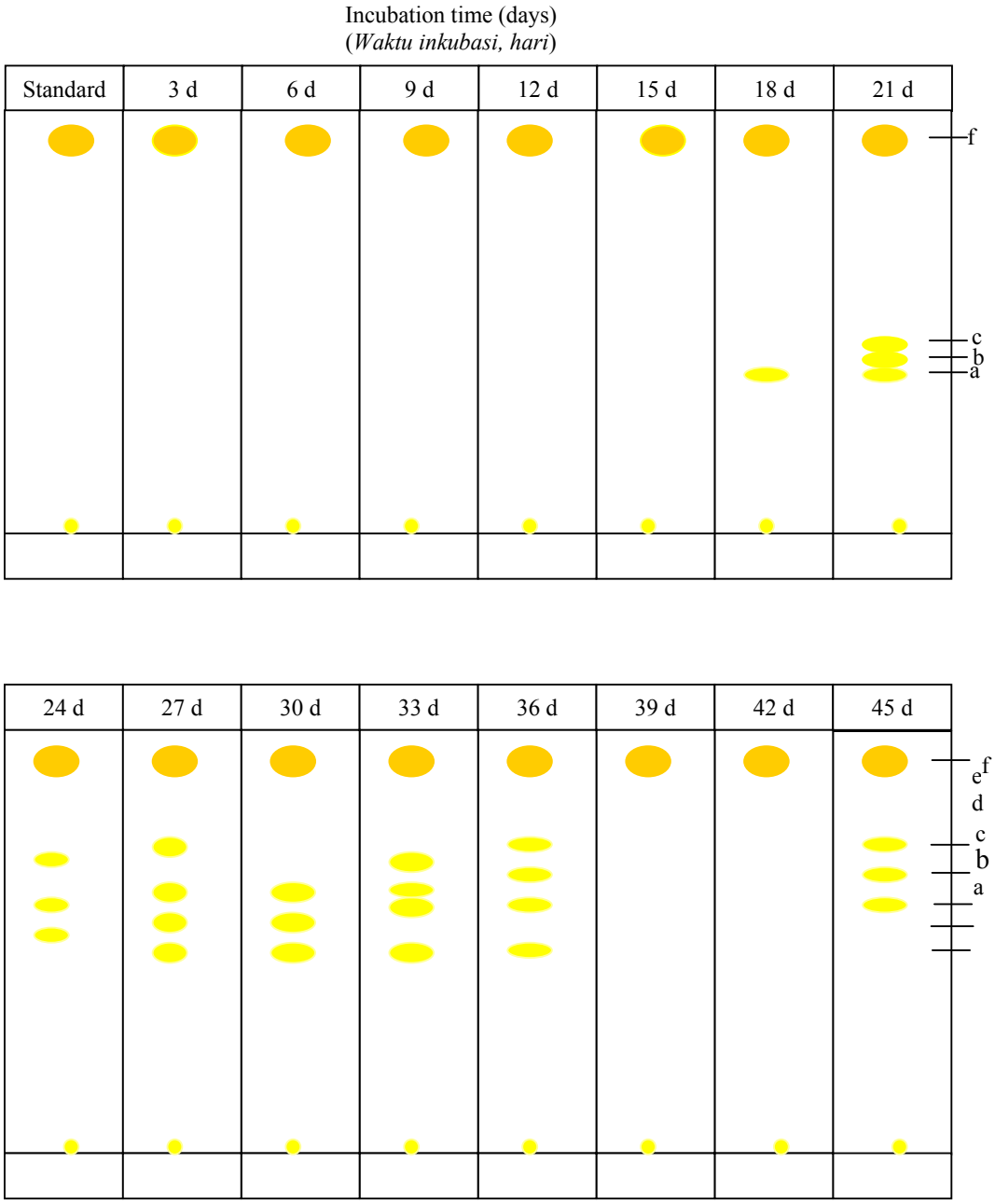

Figure 2. TLC Separation of carotenoid compounds extracted from *S. platensis* biomass grown on the best medium composition for producing carotenoids (medium III C:N:P:Mg = 1:2:0.3:0) from different incubation time (days) (node f: spot of β-carotene).

Gambar 2. Separasi TLC dari senyawa karotenoid yang diekstrak dari biomassa S. platensis *yang tumbuh pada komposisi medium terbaik untuk menghasilkan karotenoid* (*medium III C:N:P:Mg = 1:2:0.3:0*) *dari waktu inkubasi yang berbeda* (*hari*) (*node f: titik dari β-karotin*).

Table 4. Rf value of carotenoid spots isolated from biomass of *S. platensis* grown on various media composition.

Tabel 4. Nilai Rf titik karotenoid yang diisolasi dari biomassa S. platensis yang tumbuh *pada berbagai komposisi media.*

Medium *Media*	Rf value from spot (*Nilai Rf dari beberapa tempat*)					
	a	b	c	d	e	f
I	0.56	0.60	0.64	n.d	n.d	0.96
II	0.54	0.58	0.64	n.d	0.78	0.96
III	0.55	0.59	0.64	n.d	0.76	0.96
IV	0.56	0.60	0.65	n.d	0.78	0.96
VII	0.55	0.58	0.64	n.d	0.78	0.96
VIII	0.54	n.d	n.d	n.d	n.d	0.96
IX	0.53	0.58	0.64	0.69	0.79	0.96
X	0.56	0.60	0.65	0.69	0.79	0.96
XI	0.56	0.60	0.64	0.69	0.78	0.98
XII (synthetic medium of Aiba & Ogawa)	0.55	0.58	0.64	0.74	0.86	0.96

Note : Node f is a spot of ß–carotene standard; n.d : not detected
Keterangan : Node f adalah ß-karotin standard; n.d: tidak terdeteksi

Conclusions

Optimum incubation period for growing *S. platensis* in mineral media containing latex serum was eight weeks. Out of the 11 compositions of formulated low-cost media tested, the optimum medium for growth and carotenoids production of *S. platensis* was medium containing latex serum supplemented with mineral C, N, P, and Mg at ratio of 1.0: 3.0: 0.3: 0.2. This composition medium supported carotenoid in cellular biomass higher than Aiba & Ogawa synthetic medium as a reference medium. *S. platensis* on media containing latex serum contains 2-6 carotenoid compounds, in which one of them is β-carotene.

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