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Research Article

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Carotenogenesis Study of *Neurospora intermedia* N-1 in liquid substrate fermentation

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ABSTRACT

Neurospora intermedia N-1 is a fungus isolated from "oncom merah", an Indonesian red peanut cake. It has been studied for carotenoids production for food and drug colorant. Commercial production of natural carotenoids from microorganisms recently competes mainly with synthetic carotenoids by chemical procedures. Improving the efficiency of carotenoid biosynthesis can increase carotenoids production by microorganisms. Some of the cultural and environmental factors are positively affect carotenoids content of the fungal strains. The objective of this study was to evaluate the carotenogenesis of Neurospora intermedia N-1 in a liquid fermentation. The fermentation was started by optimizing the condition with light responses, then addition of variated carbon sources at 2 % w/v, co-factor Mg^{2+}, antibiotics penicillin and chloramphenicol. Carotenoid analysis was carried out by spectrophotometer UV/Vis at 480 nm and HPLC using a C_{18} column with acetonitrile: methanol: 2-propanol (85:10:5) as the mobile phase for isocratic elution. The research result showed that fermentation within 3 days incubation in dark condition and 4 days under blue light was better than 7 days in dark condition, and produced carotenoid 8.13 µg/g spores. Substrate with addition of maltose 2 % w/v and incubation at aerobic condition produced carotenoid 19.913 µg/g spores. Stimulation by co-factor Mg^{2+} in the ranges concentration below 10 mM increased the total carotenoids up to 24.31 µg/g spores, but decreased when was stimulated higher than 12 mM. Stimulation by antibiotics penicillin and chloramphenicol at concentration of 1 mg/mL increased the β-carotene content. Penicillin influenced more significant to the carotenogenesis of Neurospora intermedia N-1, particularly for β-carotene production up to 17.193 µg/g spores.

Keywords: *Neurospora intermedia*, carotenogenesis, carotenoid, co-factor Mg^{2+}, antibiotic

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INTRODUCTION

Foods and beverages are generally colored with natural or synthetic colorants as food additives [1]. Some of them are not food-grade, and some of it even dangerous to health. Therefore, natural colorants or pigments from microbial sources will be a good alternative for drugs and food colorants as long as its quality and safety are guaranteed [2, 3]. Fermentation processes for microbial pigment production on a commercial scale have been developed and now a lot of pigments are used in the food industry, such as production of ß-carotene from the fungus *Blakeslea trispora*, and production of non-carotenoid heterocyclic pigments from *Monascus* sp. which is used for food colorant [4].

Efforts have been made to reduce the production costs so that pigments produced by fermentation can be competitive with synthetic pigments or with those extracted from natural sources. Carotenoids show several beneficial functions to human beings, and largely used as colorant in foods, drinks, cosmetics and animal feed, mainly poultry and fish. In addition, many food and cosmetic industries are seeking to substitute the artificial colorants by natural-origin colorants [5].

In order to achieve simple and cost-effective carotenoids, hyper-production using carotenogenic microbes, addition of stimulants to the culture broth, and adjusting the external cultural conditions can be optimized accordingly. It has been reported that several stimulators have been studied for their enhancing effects on β-carotene production by the fungi *Blakeslea trispora* and *Phycomices blakesleeanus* which are grown under normal fermentation conditions [6].
Neurospora intermedia is a fungus isolated from Indonesian traditional food, "oncom merah", a fermented red peanut cake. The fermentation produces red-to-orange color spores, which were proved as carotenoids group. Its production on solid fermentation process has been reported elsewhere [7, 8]. Analysis of pigment extract from spores obtained from fermentation of *Neurospora intermedia* N-1 as solid substrates and it was reported that at least five carotenoid compounds were identified in spores of *Neurospora intermedia* N-1 i.e. Lycopene, Neurosporen, γ-carotene, β-carotene and Phytoene [9,10].

The objective of this study was to evaluate the carotenogenesis of *Neurospora intermedia* N-1 in liquid fermentation.

EXPERIMENTAL SECTION

Materials

Neurospora intermedia N-1 was isolated from red *oncom* samples, sampled in traditional market in Bandung area, Indonesia. Fermentation media was consisted of maltose 2%w/v, peptone 0.5%w/v, yeast extracts 0.1% w/v, co-factor Mg^{2+} was prepared from $MgSO_4.7H_2O$ (10, 12, 14 and 16 mM) and antibiotics penicillin and chloramphenicol in the concentration of 1mg/mL. Acetone was used for extraction and analysis of carotenoids.

Fermentation of *Neurospora intermedia* N-1

Neurospora intermedia N-1 was grown on agar slant and the 7-days old fungal biomass was suspended in 10 mL of sterile water, and 4% v/v of suspension spores were inoculated into 500 mL of fermentation media in 1L Erlenmeyer flask. Fermentation process was conducted according to Bhosale *et.al.* (2004) with modification. Modification was carried out by put it in dark and blue light, adding various carbon souce with concentration 2% by aerobic and an-aerobic condition with composition of air and media (10:1). The pH of media, concentration of glucose, and protein was measured with Lowry Method using visible-spectrophotometric method at λ 650 nm at beginning and end of fermentation. The weight of spore and caretonoid total was measured at λ 480 nm.

A series of fermentation flasks were prepared with addition of co-factor Mg^{2+} made from $MgSO_4.7H_2O$ with various concentrations, i.e. 10, 12, 14 and 16 mM, and antibiotics penicillin or chloramphenicol at concentration of 1mg/mL were added into each flask and incubated at 30^{o}C for 7 days. At the end of fermentation, the yellow-orange spores of *Neurospora intermedia* N-1 was harvested and pooled.

Extraction of Carotenoids

Pigment produced by *Neurospora intermedia* N-1 spores were extracted as follows: 1g of spores was extracted with 5 mL of acetone. The suspension of spores was then sonicated for 10 min, and filtered using filter paper. This process was repeated 3 times, until the residues turned to yellow pale. The pigment extract was then kept in freezer for 2-3 hours. The lipid content was separated using a syringe filter (Nylon, 0.2 µm). The separation process was carried out in a cold chamber.

Carotenoid analysis

Total carotenoids content was determined by measuring their Absorbance by UV/Vis spectrophotometer at 480 nm. Carotenoid identification was carried out by HPLC Water system C_{18} column with acetonitrile:methanol:2-propanol (85:10:5) as the mobile phase for isocratic elution. Analysis was carried out at 26^{o}C for 20 minutes, flow rates were adjusted at 2 mL/min and detection of the carotenoids by UV/Vis detector at 450 nm. Identification of carotenoids compound was compared with β-carotene standard.

RESULTS AND DISCUSSION

Liquid substrate fermentation of *Neurospora intermedia* N-1 has been carried out to study the carotenogenesis of this fungus. In the media experiment that produced the best carotenoid levels that increase in aerobic maltose, then the change in pH conditions ranging from 6 to 7, total carbohydrate substrates decreased from 5.330 g/100 mL to 1.784 g/100 mL, or reduced levels of carbohydrates as much as 3.546 g/100 mL and the protein substrate protein levels increase as much as 0.004 mg/100 mL of the initial concentration of 0.012 mg/100 mL to 0.016 mg/100 mL (Table 1).

Table 1: The influence of Carbon Source in Fermentation of *Neurospora intermedia* on Carbohydrate Level, Protein Level and pH in Media

Parameter	Carbohydrate (mg/mL)				Protein (mg/mL)				pH			
	Aerob		Anaerob		Aerob		Anaerob		Aerob		Anaerob	
	BF*)	AF*)	BF	AF	BF	AF	BF	AF	BF	AF	BF	AF
Control	2.444	0.947	2.444	1.058	0.011	0.014	0.011	0.017	6.13	8.08	6.13	7.36
Glucose	4.131	1.103	4.131	1.158	0.013	0.018	0.013	0.019	5.90	6.05	5.90	6.22
Sucrose	5.961	2.698	5.961	2.761	0.012	0.018	0.012	0.018	5.95	6.08	5.95	7.31
Maltose	5.330	1.784	5.330	2.067	0.012	0.016	0.012	0.017	6.01	6.16	6.01	7.13

**) BF: Before Fermentation, AF: After Fermentation*

Maltose is a disaccharide composed of two glucose molecules. Maltose in the fermentation by *Neurospora intermedia* first converted into a simple sugar called glucose. Results of carotenoid levels in the treatment of addition of glucose was 18.817 mg/g is not too much different and slightly lower than the addition of maltose treatment was 19.913 mg/g (Table 2). This suggests that the compounds in the form of glucose cluster is more rapidly absorbed by *Neurospora intermedia* and provide more precursors, so the availability of maltose which comprises two glucose molecules provided more energy sources and then resulted more carotenoids than glucose.

Table 2: Changes of total carotenoids content of *Neurospora intermedia* N-1 by various source of carbohydrate

Parameter	Carotenoid (µg/g)	
	Aerob	Anaerob
Control	12.710	5.774
Glucose	18.817	7.737
Sucrose	17.519	7.034
Maltose	19.913	7.814

Total effect of the carbon added to the *Neurospora intermedia* produced total carotenoids which follows the curve of the polynomial equation y = -1,3106x2 + 12,5x - 10.092. From this curve, we looked for the optimal point of carbon to produce carotenoid levels are maximized. This was related to the ratio of carbon/nitrogen (C/N), in which the media base used was 2% dextrose; peptone 0.5%; yeast extract 0.1%; and 0.04 mg / mL and the protein content of all media ranging from 0.011 to 0.013 mg/100 mL. In some publications, the content of glucose and protein are often represented as a ratio of carbohydrate/nitrogen (C/N), where this ratio determines the growth of microorganisms and productivity of primary and secondary metabolites. This ratio will be different for each strain [11].

The increase in protein content in the media before and after fermentation showed no significant effect. This was observed in the treatment of carbohydrate addition, both aerobic and anaerobic, the increase was not much different in proteins, ranged from 0.004 mg / 100 mL to 0,007 mg / 100 mL.

On this study, carotenogenesis of *Neurospora intermedia* N-1 was also stimulated by addition of co-factor Mg^{2+} and antibiotics to the fermentation media. Metal ions are involved in all aspects of microbial life. Cations such as potassium and magnesium are bulk intracellular species. Growth of mated *Blakeslea trispora* in the presence of trace Levels of Cu, Fe and Mg ions resulted in an increased rate of carotenogenesis, and gave a several-fold increase in the final yield [6,8]. In *Neurospora sp*, carotenoids production was predicted to be influenced by the co-factor of Mn^{2+} and Mg^{2+} ions.

On this study, $MgSO_4$ $7H_2O$ was used as co-factor Mg^{2+} ions with ranges concentration between 10 to 16 mM. The spores were extracted and the total carotenoids content were measured by uv/vis spectrophotometer. Analysis data of total carotenoids content was shown on Table 3. Rate of spores growth was determined by some factors such as substrates, humidity, environmental acidity degree and chemical agent. *Neurospora* growth in normal condition at substrate containing carbon, inorganic salt and biotin [6,8].

Table 3. Changes of total carotenoids content of *Neurospora intermedia* N-1 stimulated by co-factor Mg^{2+} ion (10, 12, 14 and 16 mM)

Concentration of $MgSO_4 7H_2O$ (mM)	Level of Carotenoid (mg/g spores)
Control	0,354 ± 0,000
4	0,387 ± 0,003
8	0,430 ± 0,007
12	0,496 ± 0,007
16	**0,843 ± 0.030**
20	0,419 ± 0,035

It was shown in Table 3 that co-factor Mg^{2+} ion was increased the rate of carotenogenesis of *Neurospora intermedia* N-1 when compared to the control (without co-factor), which had total carotenoids content of 17.44 µg/g spores. This was proved that the influence of co-factor Mg^{2+} ion to the carotenogenesis *Neurospora intermedia* N-1 follows the polynomial curve. Stimulation by co-factor Mg^{2+} in the ranges concentration below 10 mM, can increase the total carotenoids to 24.08 µg/g spores. However, the total carotenoids decreased when stimulated with >12 mM of Mg^{2+} . The ion acted as a co-factor in *Neurospora sp*., it was predicted for conversion of geranyl-geranyl pyrophosphate to phytoene by phytoene-synthase enzyme. Phytoene is a colorless carotenoid with only three conjugated double bonds. Several chemical agents affect carotenogenesis in a number of microbial systems [8]. These compounds, which include terpenes, ionones, amines, alkaloids and antibiotics, have been studied for their effect on carotene synthesis [10,11,12]. On this study, antibiotics penicillin and chloramphenicol (1mg/mL) were used to evaluate their effect in carotenogenesis during fermentation of *Neurospora intermedia* N-1. The spores were extracted by cold acetone to remove all lipids content and the carotene was analyzed by HPLC. Analysis of β-carotene was based on the peak area of at Rt: 13.78 ±0.2. Concentration of β -carotene of *Neurospora intermedia* N-1 that stimulated by antibiotics is presented in Figure 1.

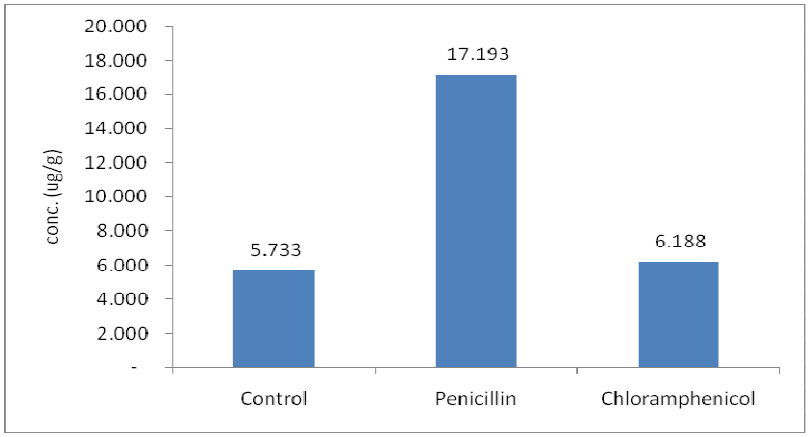
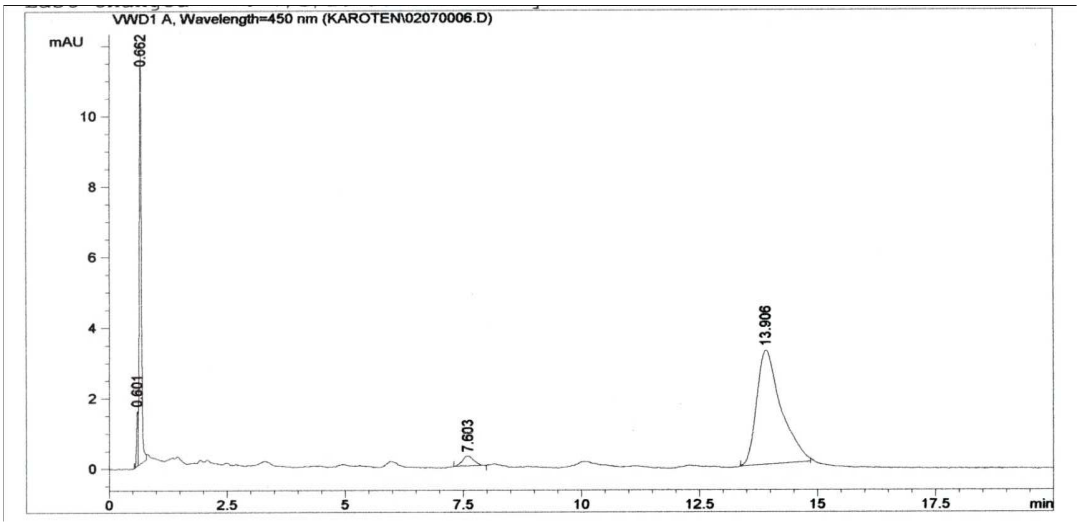


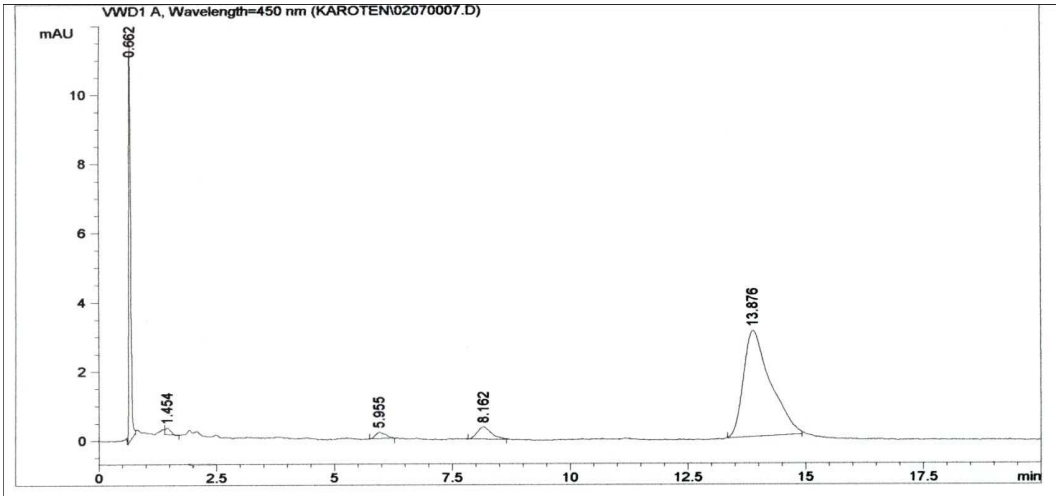
Figure 1. β-carotene of *Neurospora intermedia* N-1 stimulated by 1 mg/mL penicillin and chloramphenicol

Stimulation by antibiotics penicillin and chloramphenicol at concentration of 1 mg/ml increased the β-carotene content. Penicillin influences significantly to the carotenogenesis of *Neurospora intermedia* N-1, particularly for β-carotene biosynthesis that achieved to 17.193 µg/g spores. The growth of spores which stimulated by penicillin more abundant when compared to the control.

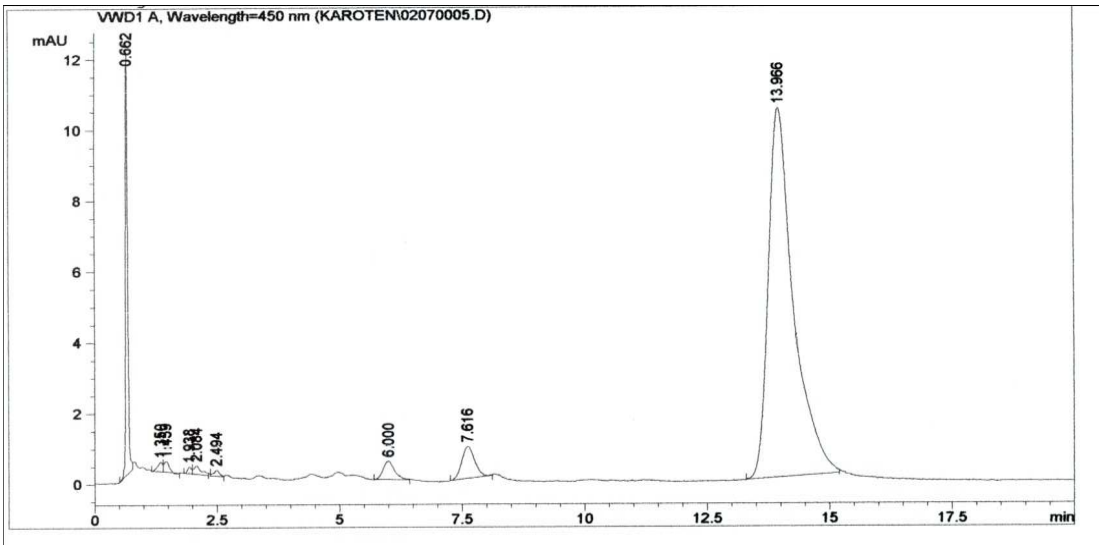
The influence of penicillin in carotenogenesis of *Neurospora intermedia* N-1 was shown also by the result of HPLC analysis (Figure 2) which has significant peak at Rt: 13.876 ±0.2.



(A)



(B)



(C)

Figure 2. HPLC chromatogram of the carotenoid of *Neurospora intermedia* N-1 from liquid substrate fermentation; (A) control (B) a β-carotene standard and (C) stimulated by penicillin

The addition of 1 mg/mL of penicillin to cultures of *Neurospora intermedia* after 24 h of growth stimulated carotenogenesis by 50% without affecting total protein and carbohydrate synthesis. Penicillin predicted exerts a stimulating effect during early stages of the isoprene biosynthetic pathway since mevalonate kinase activity was almost double in the presence of penicillin [10,11,12].

CONCLUSION

Fermentation with 3 days incubation in dark condition and 4 days under blue light was better than 7 days in dark condition, which produced carotenoid 8.13 µg/g spores. Media for *Neurospora intermedia* liquid fermentation using carbon sources maltose with the addition of 2% w/v and aerobic conditions produced the highest carotenoid that was 19.913 mg/g spores. The addition of cofactor Mg^{2+} in form $MgSO_4.7H_2O$ showed a positive influence on carotenogenesis of *Neurospora intermedia* on the addition of 12mM at the level of 24.31 µg/g spore. Production of β-carotene from *Neurospora intermedia* with the addition of antibiotics penicillin with a concentration of 1 mg / mL provides the best stimulation were analyzed using HPLC, as many as 17.193 µg /g spores.

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REFERENCES

[1] AL Branen, PM Davidson, S Salminen, JH Thorngate JH, *Food Additives.* Marcell Dekker Inc, New York, **2002**, 89-95.
[2] L Dufossé, Volume 5: Nutrition and Health, **2009,** 84.
[3] J James, *Modern Food Microbiology,* **2000,** Aspen Publisher Inc, Maryland,……
[4] L Dufossé, *Food Technol Biothechnol*, **2006,** 44, 3, 313-321.
[5] G Britton, FASEB J., **1995**, 9, 1551-1558
[6] P. Bhosale, *Appl. Microbiol. Biotechnol*., **2004**, 63:351–361
[7] D Perkins and BC Turner: *Neurospora* From Natural Population, *Experimental mycology*, 1**988,** 12, 91-131.
[8] U Mitzka-Schnabel, *Pure and Appl Chem*, **1985**, **57**, 667-669
[9] S Priatni, L Leenawaty, Heriyanto, M Singgih, and T Gusdinar, Proceedings of NP-SEA, Malang, **2010**, 92-95
[10] G Sandmann, S Takaichi, PD Fraser, *Phytochemistry*, **2008**, 69 2886-2890.
[11] C Sieiro, M Poza, T de Miguel, TG Villa, *Int. Microbiol*, **2003,** 6, 11-16
[12] HG Desai and VV Modi VV, *Phytochemistry*, **1977**, 16, 1373-1376.